Studies of Vascular Endothelial Growth Factor - Related Peptides in the Rat Testis

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ABSTRACT

Vascular endothelial growth factor (VEGF) family is a group of structurally related peptides important in controlling vascular development and functions. We have previously demonstrated the expression of VEGF-A and its receptors (VEGFR-1 and VEGFR-2) in the testis. This study aims at determining whether other members of VEGF family are also present, thus providing the basis for further understanding their role in the vascular control of the testis. Hormonal regulation of these members is also examined by injection of single dose of human chorionic gonadotrophin (hCG) to adult rats.

Using RT-PCR, mRNA of all VEGF family members were shown to be expressed in rat testes, primary Leydig and Sertoli cells, and testicular cell lines from rat (LC540, R2C) and mouse (MLTC-1, TM3, TM4). Western immunoblotting of tissue and cell lysates revealed positive bands (27 kDa, 21 kDa and 15 kDa) of VEGF-A_{188}, VEGF-A_{164} and VEGF-A_{120}; and bands (35 kDa and 24 kDa) of VEGF-B_{186} and VEGF-B_{167}. For VEGF-C, positive band was identified at 27 kDa, VEGF-D at 46, 37, 23 and 16 kDa and PlGF (placenta growth factor) at 27 kDa. Variations on signal intensity of protein bands were observed in different samples and these could be related to their relative abundance and levels of processing in different cell types. Immunostaining showed strong immunoreactivity in Leydig cells, and

weak in Sertoli cells and vascular smooth muscle cells. Following the destruction of Leydig cells by ethane dimethane sulphonate (EDS) treatment, most immunoreactivity in the testicular interstitium disappeared and resulted in decreases in the levels of VEGF-A_{164} and VEGF-A_{120} isoforms, VEGF-B_{186} and VEGF-B_{167} isoforms, but increases in the levels of VEGF-D (mRNA and 16 kDa isoform) and PlGF. Chronic Leydig cell suppression by an eight-week treatment with exogenous testosterone released from subcutaneous silastic implants showed similar effect to EDS treatment on the expression of VEGF-A_{164} isoform. A single subcutaneous injection of 100 IU hCG was shown to increase the expression of VEGF-A_{164} in testes of normal rats and those receiving testosterone implants. This was also accompanied by a return of VEGF immunoreactivity in Leydig cells.

In conclusion, expression of various new VEGF family members was demonstrated in the testis (mainly found in Leydig cells and Sertoli cells). Leydig cell was demonstrated to be a major source of VEGF-A_{164} and VEGF-A_{120} isoforms, VEGF-B_{186} and VEGF-B_{167} isoforms. Expression of VEGF-D (mRNA and 16 kDa isoform) and PlGF was demonstrated to be androgen repressible. Besides, expression of VEGF-A_{164} was demonstrated to be hormonally regulated by hCG.

摘　要

血管內皮生長素 (vascular endothelial growth factor, VEGF) 家庭是一群結構相似的縮氨酸，對血管生長及其功能有著重要影響。我們以往已經證實血管內皮生長素甲型 (VEGF-A) 及其受體一型和二型 (VEGF receptor, VEGFR-1 和 VEGFR-2) 是存在於睾丸中。是項研究旨在測定是否有其他的血管內皮生長素家庭成員存在，以致提供基礎來認識睾丸中血管生長的控制機制。我們也會注射人絨毛膜促性腺激素 (human chorionic gonadotrophin, hCG) 到成年大白鼠來考察各血管內皮生長素家庭成員的荷爾蒙控制機制。

我們利用逆轉腖-聚合腖連鎖反應 (reverse transcriptase-polymerase chain reaction) 錄得所有的血管內皮生長素家庭成員的信使核糖核酸 (mRNA)，都能於大白鼠睾丸、萊迪希氏細胞 (Leydig cell)、謝爾托立氏細胞 (Sertoli cell)、大白鼠細胞株 (LC540 和 R2C) 及小白鼠細胞株 (MLTC-1, TM3 和 TM4) 中找到。利用韋斯頓免疫印跡術 (Western immunoblotting) 在組織及細胞的溶解產物中找到血管內皮生長素甲型之眾等型 (VEGF-A_{188}, VEGF-A_{164} 和 VEGF-A_{120})，其分子量分別爲 27、21 及 15 千道爾頓 (kilo Dalton, kDa)；及血管內皮生長素乙型 (VEGF-B) 之眾等型 (VEGF-B_{186} 和 VEGF-B_{167})，其分子量分別爲 35 及 24 千道爾頓。血管內皮生長素丙型 (VEGF-C) 的分子量爲 27 千道爾頓。血管內皮生長素丁型 (VEGF-D) 的分子量分別爲 46、37、23 及 16 千道爾頓. 胎盤生長素(PlGF) 的分子量則爲 27 千道爾頓。各種血管內皮生長素的蛋白質信號於不同樣本中有

著不同的強度變化，這可能是與它們不同的相對豐度和於不同細胞類型中的變化過程有關。免疫染色法顯示萊迪希氏細胞有著很強的信號，而謝爾托立氏細胞和維管平滑肌細胞 (vascular smooth muscle cells) 則有較弱的信號。以乙烷 1，2 二甲烷磺酸鹽破壞睾丸中的萊迪希氏細胞後，造成大部分的免疫染色法信號於睾丸間隙中消失，以及降低了血管內皮生長素甲型之眾等型 (VEGF-A_{164} 和 VEGF-A_{120})，及血管內皮生長素乙型之眾等型 (VEGF-B_{186} 和 VEGF-B_{167}) 的信號，卻提升了血管內皮生長素丁型 (VEGF-D) 和胎盤生長素 (PlGF) 的信號。當使用外源性的睾丸酮皮下包埋八週達到慢性萊迪希氏細胞抑制後發覺與乙烷 1，2 二甲烷磺酸鹽於血管內皮生長素甲型 (VEGF-A_{164}) 所造成的影響相似。一次注射 100 國際單位(IU)的人絨毛膜促性腺激素亦證實能提升血管內皮生長素甲型 (VEGF-A_{164}) 在正常或帶有睾丸酮皮下包埋之大白鼠的表顯。這也同時恢復在萊迪希氏細胞中的血管內皮生長素免疫染色法信號。

總括而言，在睾丸中能找到各種新的血管內皮生長素家庭成員 (多數於萊迪希氏細胞、謝爾托立氏細胞中找到)。我們證實了萊迪希氏細胞是血管內皮生長素甲型之眾等型 (VEGF-A_{164} 及 VEGF-A_{120}) 和血管內皮生長素乙型之眾等型 (VEGF-B_{186} 及 VEGF-B_{167}) 的主要來源。我們也證實血管內皮生長素丁型 (信使核糖核酸和 16 千道爾頓等型) 和胎盤生長素 (PlGF) 是可被雄激素抑制的。此外，我們證實了血管內皮生長素甲型 (VEGF-A_{164}) 是可被荷爾蒙 (人絨毛膜促性腺激素) 所控制的。

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1. Introduction

1.1 General review of angiogenesis

Vasculogenesis and angiogenesis are the fundamental processes by which new blood vessels are formed (Carmeliet, 2000; Risau, 1997; Risau & Flamme, 1995; Vailhe *et al.*, 2001). Vasculogenesis is defined as the differentiation of precursor cell, angioblasts, into endothelial cells and the *de novo* formation of a primitive vascular network, whereas angiogenesis is defined as the growth of new capillaries from pre-existing blood vessels (Risau, 1997; Vailhe *et al.*, 2001).

While vasculogenesis is mainly found in early embryogenesis, angiogenesis occurs not only in embryos but also is an important component of tissue growth and development during postnatal life. However in adults, angiogenesis rarely occurs under physiological conditions except in the female reproductive system where it forms an integral part of follicular development, corpus luteum formation, repair of the endometrium during the menstrual cycle, formation of the placenta and development of the breast during pregnancy. Angiogenesis in adults is most commonly associated with pathological conditions such as wound healing, inflammation, diabetic retinopathy, psoriasis, rheumatoid arthritis, and solid tumor growth (Breier *et al.*, 1997; Edward *et al.*, 2001; Folkman, 1995; Pepper, 1997; Vailhe *et al.*, 2001)

1.2 Vascular endothelial growth factors (VEGFs)

Vascular endothelial growth factors (VEGFs) comprise a group of structurally and functionally related growth factors that modulate many important physiological functions of endothelial cells. Currently, five different mammalian VEGFs (VEGF-A, PlGF, VEGF-B, VEGF-C and VEGF-D) have been identified and they all show unique temporal and spatial expression patterns, receptor specificity and function (Li & Eriksson, 2001).

The VEGF family members are secreted dimeric glycoproteins, all of which contain the characteristic regularly-spaced eight cysteine residues in the VEGF homology domain (Fig.1.1). These eight invariant cysteine residues are shown to be involved in inter- and intramolecular disulfide bonds in several of these dimeric growth factors (Li & Eriksson, 2001; Veikkola & Alitalo, 1999).

The VEGFs play pivotal roles in the formation of the vascular system during embryonic development, in the regulation of capillary growth in normal and pathological conditions in adults, and in the maintenance of the normal vasculature (Li & Eriksson, 2001; Olofsson et al., 1999).

1.2.1 VEGF-A

The most potent and well-studied angiogenic factor is vascular endothelial growth factor A (VEGF-A) which was discovered more than a decade ago (Aase,

2001; Li & Eriksson, 2001). The gene encoding human VEGF-A produces six different transcripts by alternative splicing of the eight exons in the mRNA, VEGF-A_{110}, VEGF-A_{121}, VEGF-A_{145}, VEGF-A_{165}, VEGF-A_{189} and VEGF-A_{206} (Houck *et al.*, 1991; Lei *et al.*, 1998; Leung *et al.*, 1989; Poltorak *et al.*, 1997). On the other hand, four variants from the mouse homologue have been found, VEGF-A_{120}, VEGF-A_{144}, VEGF-A_{164} and VEGF-A_{188} (Shima *et al.*, 1996; Sugihara *et al.*, 1998).

The biologically active molecules are glycosylated disulphide-linked dimers, which display different biological properties. For example, the shortest isoforms, VEGF-A_{115} and VEGF-$A_{120/121}$ are freely secreted whereas the others are sequestered on the cell surface or the extracellular matrix due to binding to heparin sulfate proteoglycans (Poltorak *et al.*, 2000; Sugihara *et al.*, 1998). The predominant forms of VEGF-A are VEGF-$A_{120/121}$, VEGF-$A_{164/165}$ and VEGF-$A_{188/189}$, whereas the others are comparatively rare (Robinson & Stringer, 2001). VEGF-A mediates its functions by binding and activating receptors located on the cell surface, VEGF receptor-1 (VEGFR-1/ flt-1), VEGFR-2 (flk-1/KDR) and neuropilin-1 and -2 (NP-1 and -2) (Neufeld *et al.*, 1999) (Fig.1.2).

VEGF-A is recognized as a major hypoxia-inducible growth factor for vascular endothelial cells and it is upregulated under hypoxic conditions and downregulated under hyperoxic conditions (Alon *et al.*, 1995; Shweiki *et al.*, 1992). Studies have

also indicated that VEGF is hormonally-regulated by steroid hormones (Laitinen *et al.*, 1997; Neulen *et al.*, 1995, 1998; Ruohola *et al.*, 1999).

1.2.2 PlGF

The second growth factor in the VEGF family is called placenta growth factor (PlGF) because it was originally isolated from a human placenta cDNA library (Aase, 2001; Maglione *et al.*, 1991). It is about 50% homologous to VEGF-A (Maglione *et al.*, 1991). Similar to VEGF-A, PlGF is a glycosylated, secreted disulphide-linked dimer. In human, three PlGF isoforms have been reported and they are formed from alternative splicing of PlGF gene, PlGF-1 ($PlGF_{131}$), PlGF-2 ($PlGF_{152}$) and PlGF-3 ($PlGF_{221}$) (Cao *et al.*, 1997; Hauser & Weich, 1993; Maglione *et al.*, 1993). Only PlGF-2 binds heparin with high affinity suggesting that this isoform is sequestered on the cell surface/ extracellular matrix (Hauser & Weich, 1993), while the other two isoforms are freely secreted. In rodents, only PlGF-2 homologue has been found with a native peptide of 158 amino acid (AA) residues and a mature peptide (after removal of signal sequence) of 135 AA (DiPalma *et al.*, 1996; DiSalvo *et al*, 1995). All variants of PlGF bind VEGFR-1, but only the heparin binding PlGF-2 bind NP-1 (Migdal *et al.*, 1998; Park *et al.*, 1994). None of the isoforms bind VEGFR-2 (Park *et al.*, 1994).

PlGF overlaps in the expression with VEGF-A both in normal tissues, and in

many tumours. In fact naturally existing heterodimers of PlGF/VEGF were purified from a rat glioma cell line (DiSalvo *et al.*, 1995). Later, recombinant heterodimers were also purified and shown to induce binding to VEGFR-2 (Cao *et al.*, 1996). In contrast to VEGF-A, PlGF has been shown to be downregulated under hypoxic conditions (Gleadle *et al.*, 1995).

1.2.3 VEGF-B

VEGF-B or VEGF-related factor (VRF) was discovered in 1996 (Olofsson *et al.*, 1996a). It is closely related to VEGF-A, with a homology of about 43% (Olofsson *et al.*, 1996a). The mouse and human VEGF-B genes are almost identical and are composed of seven exons (Olofsson *et al.*, 1996b). There are two isoforms of VEGF-B, VEGF-B_{167} and VEGF-B_{186}, produced by alternative splicing of the acceptor site in exon 6. Difference in the carboxy-terminals of the two isoforms affects their biochemical and biological features. The shorter variant contains a cell-associating, heparin-binding carboxy-terminus resembling that of VEGF-A, whereas the carboxy-terminal region of the longer variant is hydrophobic and freely secreted (Aase, 2001; Olofsson *et al.*, 1996a, b) (Fig.1.1).

VEGF-B shares many features of VEGF-A, such as its disulphide-linked dimerisation and binding to the VEGFR-1 and NP-1, but unlike VEGF-A, VEGF-B is not affected by hypoxia and hormone and does not bind VEGFR-2 (Enholm *et al.*,

1997; Laitinen *et al.*, 1997; Makinen *et al.*, 1999; Olofsson *et al.*, 1998).

1.2.4 VEGF-C and VEGF-D

VEGF-C and VEGF-D differ in many aspects from VEGF-A, PlGF and VEGF-B. They are mainly thought of as being important mainly for lymphatic endothelium and lymphangiogenesis and constitute a subgroup based on their unique amino- and carboxy-terminal regions flanking the VEGF homology domain (Fig.1.1) (Aase, 2001; Joukov *et al.*, 1996; Joukov *et al.*, 1997b; Lee *et al.*, 1996; Orlandini *et al.*, 1996; Yamada *et al.*, 1997). Their carboxy-terminal regions contain a repetitive pattern of cysteine residues with homology to a component in the silk produced by the silk larvae, *Chironomus tentans* (Chilov *et al.*, 1997). VEGF-C and VEGF-D are synthesised as pre-proproteins where the carboxy-terminal parts are cleaved off upon secretion, but remain bound via disulphide bonds. The proproteins bind to VEGFR-3 (flt-4), a receptor exclusively expressed on lymphatic endothelium, whereas binding to VEGFR-2 requires proteolytic processing of the amino-terminal regions (Joukov *et al.*, 1997a; Kukk *et al.*, 1996).

Unlike VEGF-A, the expression of VEGF-C does not appear to be regulated by hypoxia (Enholm *et al.*, 1997), but is increased in response to proinflammatory cytokines suggesting a role in inflammatory responses (Ristimaki *et al.*, 1998). It has also been known that VEGF-C is hormonally down-regulated by steroid

hormones (Laitinen *et al.*, 1997). VEGF-C is likely to play a dual role, both as an angiogenic and lymphangiogenic growth factor; depending on the spatio-temporal expression of its receptors (Cao *et al.*, 1998). VEGF-D has been shown to be angiogenic as well as serving as a mitogen for both fibroblasts and endothelial cells, although it is less potent than VEGF-A (Achen *et al.*, 1998; Marconcini *et al.*, 1999; Orlandini *et al.*, 1996). Interestingly, mouse VEGF-D does not bind mouse VEGFR-2, suggesting that the biological functions of VEGF-D differ in mouse and man, and that VEGF-D interaction with VEGFR-2 is not crucial for normal development (Baldwin *et al.*, 2001).

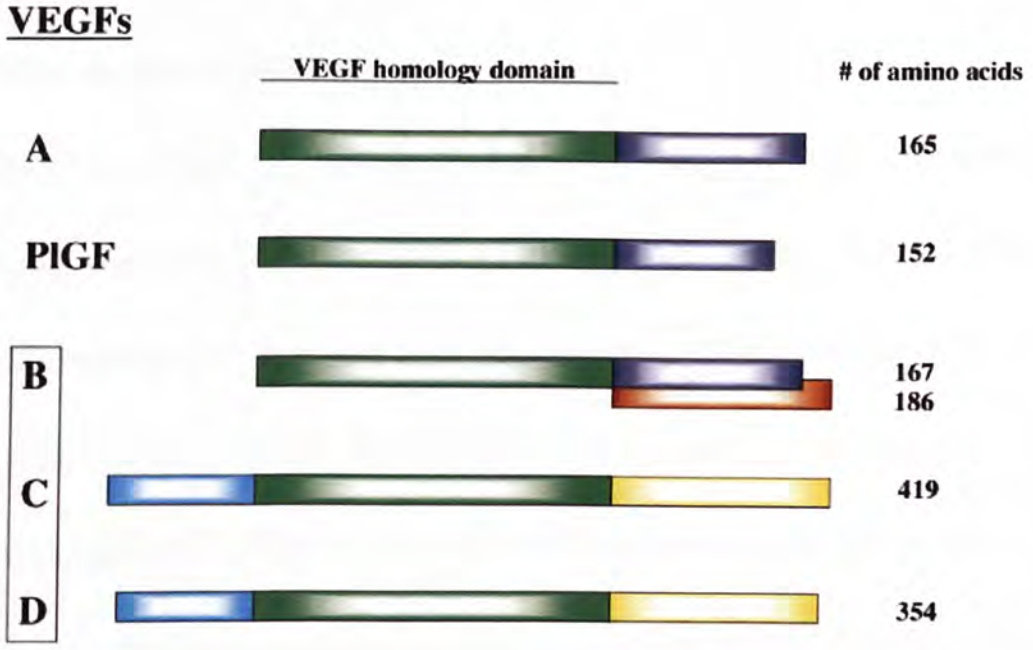


Fig.1.1 Schematic illustration of the domain structures of the currently known members of the VEGF family, including the novel VEGF family members, VEGF-B, VEGF-C and VEGF-D. The different domain structures include the VEGF homology domain (in green), the C-terminal regions containing the basic heparin-binding domains in some spliced isoforms of VEGF, PlGF and VEGF-B (in dark blue), the unique C-terminal domain in VEGF-B186 (in red), the N-terminal propeptide domains (in light blue), and silk domain-containing C-terminal propeptides in VEGF-C and D (in yellow). Note that the different domains are not drawn in scale. The numbers to the right refer to the number of amino acids in the proteins shown. (Adapted from Li & Eriksson, 2001)

1.3 VEGF receptors (VEGFRs)

VEGFs exert their functions via binding to cell surface receptors and thereby transducing their signals into the cell (Fig.1.2). The VEGFs bind to two different types of receptors, the VEGF receptors (VEGFRs) and neuropilins (NP). VEGFRs are receptor tyrosine kinases that consist of an extracellular domain of seven immunoglobulin-like (Ig) repeats, a hydrophobic transmembrane domain that anchors the receptor in the cell membrane, a juxtamembrane domain and a protein kinase domain that is split into two halves. The dimeric VEGFs bind two receptors simultaneously; thus forming a stable receptor dimer that becomes activated by the subsequent autophosphorylation, i.e. one receptor subunit phosphorylates the other (Aase, 2001; Veikkola & Alitalo, 1999).

1.3.1 VEGFR-1 (or flt-1)

VEGFR-1, or flt-1 (fms-like tyrosine kinase), is the receptor for VEGF-A, PlGF and VEGF-B (Aase, 2001; de Vries *et al.*, 1992; Olofsson *et al.*, 1998; Park *et al.*, 1994; Shibuya *et al.*, 1990). Just as many of the VEGFs, the VEGFR-1 gene gives rise to two different isoforms; a full-length receptor and an extracellular form called soluble VEGFR-1 (sVEGFR-1) (Kendall *et al.*, 1996). This soluble receptor is abundantly expressed in the placenta and can form heterodimers with VEGFR-2

(Kendall *et al.*, 1996).

The kinase activity of VEGFR-1 is much lower than that of other fms tyrosine kinases and the receptors for the PDGFs (Sawano *et al.*, 1996; Shibuya *et al.*, 1999). The weak kinase activity could be a result of unstable VEGFR dimers, tight regulation by tyrosine phosphatases or few phosphorylation sites (Shibuya *et al.*, 1999).

One interesting fact with VEGFR-1 is that the catalytic cytoplasmic domain is not essential for proper cardiovascular development, thus signalling from VEGFR-1 seems to be insignificant during embryogenesis and the role of VEGFR-1 might be that of a sink, where excess VEGF-A is drained (Hiratsuka *et al.*, 1998). However, VEGF-A- or PlGF-induced monocyte migration was suppressed in mice lacking the intracellular tyrosin kinase domain of VEGFR-1.

1.3.2 VEGFR-2 (or flk-1)

The second receptor was originally called KDR (kinase insert-domain containing receptor) for the human protein and flk-1 (fetal liver kinase 1) for the mouse homologue (Aase, 2001; Matthews *et al.*, 1991; Terman *et al.*, 1991). VEGFR-2 binds VEGF-A, VEGF-C and VEGF-D (Achen *et al.*, 1998; Joukov *et al.*, 1996; Terman *et al.*, 1992). VEGFR-2 mRNA is first detected in presumptive mesodermal yolk-sac blood island progenitors and then in the primitive endothelium surrounding the blood islands, thus VEGFR-2 seems to play an important role during

vasculogenesis (Shalaby *et al.*, 1995). The overall structure and binding properties are highly related to VEGFR-1 (Fuh *et al.*, 1998; Shibuya *et al.*, 1999).

In vitro studies have shown that VEGFR-1 and VEGFR-2 respond differently to VEGF-A stimulation. Both receptors induced cell migration, but mitogenic signals were only transduced via VEGFR-2 (Barleon *et al.*, 1996; Seetharam *et al.*, 1995; Soker *et al.*, 1998; Waltenberger *et al.*, 1994; Yoshida *et al.*, 1996). Also, VEGF-A-induced vascular permeability occurs primarily via VEGFR-2 (Hiratsuka *et al.*, 1998; Joukov *et al.*, 1998).

1.3.3 VEGFR-3 (flt-4)

VEGFR-3 (flt-4) is a receptor for VEGF-C and VEGF-D and differs from the other VEGF receptors is that it has to be proteolytically processed before it can bind to its ligands (Aase, 2001; Achen *et al.*, 1998; Joukov *et al.*, 1996; Pajusola *et al.*, 1992; Pajusola *et al.*, 1994). Early in mouse development, VEGFR-3 is expressed in the cardinal vein and in the angioblasts of the head mesenchyme but as embryogenesis continues VEGFR-3 expression becomes restricted to the lymphatic endothelium (Kaipainen *et al.*, 1995). In adults, VEGFR-3 is found in the lymphatic endothelium of the lung, mesenterium and tonsil, while vascular endothelium is negative (Kaipainen *et al.*, 1995). In spite of the documented restriction to lymphatic vessels, recent data show that VEGFR-3 is expressed both in the blood and

lymphatic vessels in the nasal mucosa of human adults and in the fenestrated capillaries of several organs (Partanen *et al.*, 2000; Saaristo *et al.*, 2000).

In line with the angiogenic capacity of VEGF-C and VEGF-D, VEGFR-3 was shown to play a role in the development of the cardiovascular system before lymphatic vessels appear (Dumont *et al.*, 1998). Vasculogenesis was not affected in VEGFR-3 deficient embryos, nor was sprouting angiogenesis, but VEGFR-3 signalling was shown to be important for vascular remodelling and maturation of the primitive vascular network (Aase, 2001; Dumont *et al.*, 1998; Veikkola & Alitalo, 1999).

In human, the VEGFR-3 gene encodes two polypeptides (a long form and a short form) that differ at their C-terminus due to alternative splicing (Galland *et al.*, 1993; Pajusola *et al.*, 1993). The long form is the predominant form in most tissues. It is interesting to note that an endogenous retroviral genome appears responsible for the short isoform in humans, but this form is missing from mice (Hughes, 2001).

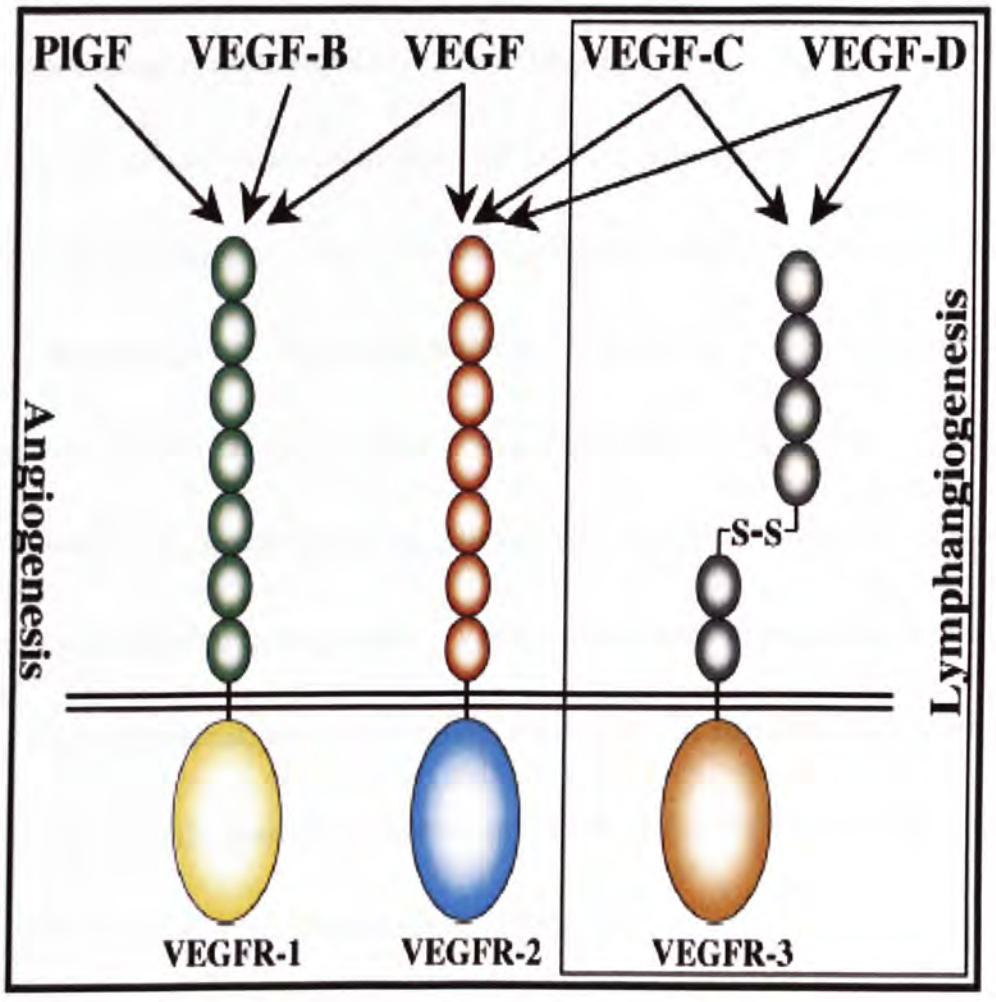


Fig.1.2 Schematic illustration of the binding of VEGF family members to VEGF receptors 1, 2 and 3. Ligand activation of the receptors induces a variety of effects in the receptor-expressing endothelial cells including growth, survival, permeability, migration and vessel remodelling. VEGFR-3 and its two ligands, VEGF-C and VEGF-D, are specifically involved in lymphangiogenesis while all VEGFs and their receptors are implicated in angiogenesis. The different interactions of VEGFs with soluble VEGFR-1, the co-receptor NP-1, and with heparan sulfate proteoglycans are not shown. (Adapted from Li & Eriksson, 2001)

1.4 Hormonal regulation of VEGFs by LH/hCG

Human chorionic gonadotrophin (hCG) is a glycoprotein hormone normally secreted by the placenta. However, it is structurally and biologically closely related to the pituitary-derived luteinizing hormone (LH), hCG binds to the same receptor and acts as a potent LH agonist (Gharib *et al.*, 1990; Rulli *et al.*, 2003).

Evidence is accumulating to indicate that VEGF is involved in mediating hormonally-regulated angiogenesis. VEGF is expressed in many endocrine glands (e.g. thyroid, pancreas, adrenal, pituitary and testis) and hormone-responsive tissues like the uterus, fallopian tube, placenta and breast (Fujimoto *et al.*, 1998; Gordon *et al.*, 1996; Toi *et al.*, 1995; Winther *et al.*, 1999).

In the ovary, Neulen *et al.* (1995) demonstrated that the VEGF gene expression in cultured luteinized granulosa cells was stimulated by LH/hCG in both time- and dose-dependent manner. These cells were obtained from human ovarian follicles around the time of ovulation when a marked increase in follicular angiogenesis occurs. Later, Neulen *et al.* (1998) showed VEGF secreted from human luteinized granulosa cells is hCG dependent and suggested that vascularization of the corpus luteum is caused by hCG-induced VEGF release. Laitinen *et al.* (1997) demonstrated that primary cultures of human granulosa-luteal (GL) cells exposed to recombinant human

FSH (rhFSH) significantly increased the levels of VEGF mRNA, however, VEGF-C mRNA levels were down-regulated and VEGF-B mRNA levels seemed more or less unchanged. Studies also presented that VEGF protein was expressed throughout the luteal phase, and hence it might play a role in the cyclic growth of ovarian follicles and corpus luteum development and maintenance, and mediating ovarian angiogenesis. Furthermore the expression and secretion of VEGF were induced by both FSH and LH/hCG receptor-activated pathways (Geva & Jaffe, 2000; Wulff *et al.*, 2001).

Gonadotrophin suppression by gonadotrophin releasing hormone (GnRH) antagonist treatment significantly lowered the increase in VEGF mRNA levels in transplanted ovaries of immature rats (Dissen *et al.*, 1994), and this could not be compensated by injecting these animals with PMSG (pregnant mare serum gonadotrophin), suggesting the activity of LH might be more important. Finally, conditioned medium from gonadotrophin-treated human ovarian carcinoma cells was shown to be mitogenic to bovine endothelial cells and this activity could only be blocked by neutralizing antibodies against LH or VEGF (Schiffenbauer *et al.*, 1997). Using RT-PCR, both LH and FSH were shown to produce a dose-dependent stimulation of VEGF expression in these cultured human ovarian cancer spheroids. Based on the above findings of a predominant LH effect in stimulating VEGF

expression in the ovary, it would be tempting to speculate that LH might have a similar action in the testis to upregulate VEGF expression in Leydig cells, and this was confirmed by a recent study demonstrated by Haggstrom Rudolfsson *et al.* (2003) that testicular VEGF secretion was increased by hCG stimulation of Leydig cells and that VEGF, through effects mediated via VEGFR-2, regulated endothelial cell proliferation in the rat testis.

LH/hCG is known to cause vascular changes in the testis. Following a single subcutaneous injection of 50-100 IU hCG into adult rats, vascular permeability increased together with an accumulation of interstitial fluid and a rise in blood flow (Setchell & Sharpe, 1981). The same dose of hCG (100 IU) has recently been shown to stimulate endothelial cell proliferation in the testicular vasculature (Au *et al.*, 1996; Collin & Bergh, 1996), with the maximum changes occurring at 2 days post-injection. The angiogenic response (Au *et al.*, 1996; Collin & Bergh, 1996) and the permeability increase (Setchell & Rommerts, 1985; Collin & Bergh, 1996) are dependent on factors originating from the Leydig cells as the changes are completely abolished following the depletion of Leydig cells by ethane dimethane sulphonate treatment. This would be expected since Leydig cells represent the only cell type in the testis that binds LH/hCG (Mendelson *et al.*, 1975). The Leydig cell product that could best fulfill such a role is VEGF as it can induce vascular hyperpermeability,

endothelial cell proliferation, and an increase in blood flow (Lopez *et al.*, 1997; Ni *et al.*, 1997; Tilton *et al.*, 1999). In the view of above findings, the expression and regulation of VEGFs in the testis by LH/hCG are worth for further exploring.

1.5 General review of the testis

1.5.1 Structure and function of the testis

The testes are oval, paired structures suspended by the spermatic cords and housed within the scrotum. Each testis is surrounded by a membranous capsule which is made up of three distinct layers, namely tunica vasculosa, tunica albuginea and tunica vaginalis. The testis parenchyma is structurally and functionally divided into two compartments – the seminiferous tubules and the intertubular tissue (or interstitium). Lining the seminiferous tubules is a compound epithelium consisting of germ cells and Sertoli cells. Production of spermatozoa occurs within these tubules in a process known as spermatogenesis. The interstitium contains lymphatic vessel, blood vessels, variable amount of connective tissues and a variety cell types including Leydig cells, macrophages, fibroblasts and mast cell.

The testes serve two major functions in adults, namely the production of spermatozoa and the secretion of androgens, mainly testosterone. These functions are known to be regulated primarily by the two gonadotrophins produced by the anterior pituitary – follicle stimulating hormone (FSH) and luteinizing hormone (LH).

The release of these two hormones is in turn under the control of gonadotrophin releasing hormone (GnRH) produced by the hypothalamus. FSH acts specifically on the Sertoli cells to support the process of spermatogenesis. LH acts on the Leydig cells to stimulate androgen production which is needed for the development and maintenance of the spermatogenic process as well as the secondary sexual characteristics. In addition to the hypothalamic-pituitary control of testicular functions, there is increasing evidence to indicate that hormone actions within the testis are modulated by numerous paracrine and autocrine factors produced locally as means of cell-cell interactions (Huhtaniemi & Toppari, 1995; Jegou & Pinau, 1995).

1.5.2 Testicular vasculature

In mammalian testes, the vascular system possesses several peculiar structural organizations which indicate that it has important control over the function of the testis (Bergh *et al.*, 1993; Setchell, *et al.*, 1994). These include a long unbranched testicular artery, the presence of a peritubular and an intertubular capillary network, the lining of the testicular vasculature by an unfenestrated endothelium and the failure of the capillaries to penetrate the seminiferous tubules. Due to the haemodynamic properties of a long unbranched blood vessel, the rate of blood flow to the testis is intrinsically limited by the testicular artery. An unfenestrated endothelium may hinder the passage of important macromolecules like the gonadotrophins, from blood

to their target cells in the extravascular tissues. In addition, the above problems could be compounded by the fact that the seminiferous tubules which make up the bulk of the testicular tissues and possess the actively dividing and differentiating germ cells are avascular. Since oxygen has to diffuse from the capillaries lying in the intertubular areas to the seminiferous tubules, the centre of these tubules has been shown to be at the brink of hypoxia (Setchell, 1978). This is especially true when the metabolism within the testis is increased (e.g. elevated testicular temperature) without being matched by a corresponding rise in blood flow. In the view of the limitations imposed by the anatomical arrangement of the testicular vasculature, a well-developed and maintained blood supply appears to extremely important to the normal activities within the testis and may provide the necessary local control of testicular function. In the vascular control within the testis, besides the acute effects from the alteration of the vascular permeability, and the regulation of blood flow by vasodilation or vasoconstriction, there is a medium- to long-term regulation by controlling the blood vessel formation or angiogenesis.

1.5.3 Testicular angiogenesis

It remains to be elucidated how the angiogenic process in the testis is initiated and controlled. However, quite a number of angiogenic factors have been found in mammalian testes, like acidic fibroblast growth factor (aFGF), basic fibroblast growth

factor (bFGF), platelet-drived growth factor (PDGF) and vascular endothelial growth factor (VEGF) (Ergun *et al.*, 1997; Gnessi *et al.*, 1995; Laslett *et al.*, 1997; Murono *et al.*, 1992; Norrby, 1997; Zheng *et al.*, 1990). Among them, only VEGF has the signal peptide required for secretion and also it is mitogenic only to endothelial cells (Ferrara, 1996; Ferrara & Davis-Smyth, 1997; Leung *et al.*, 1989). So it makes VEGF a very attractive candidate for the study of how the angiogenic process is being regulated within the testis.

1.6 Localization of VEGF and VEGF receptors in the testis

The first report indicating the presence of VEGF in the testis was the work done in the mouse where VEGF transcripts were localized by *in situ* hybridization in the testicular interstitium, most likely associated with the Leydig cells (Shweiki *et al.*, 1993). Wan *et al.* (1996) were the first to report on the cloning of VEGF gene in the Leydig cells. Korpelainen *et al.* (1998) demonstrated that VEGF and VEGF receptors were expressed in the testis and epididymis where overexpression of VEGF caused spermatogenic arrest, epithelial hyperplasia and infertility. Using immunohistochemical staining, Au *et al.* (1997) and Collin & Bergh (1996) demonstrated that VEGF immunoreactivity was predominantly associated with the Leydig cells, though lower levels of expression were also found in the Sertoli cells and vascular smooth muscles. In line with the above findings, Ergun *et al.* (1997)

used RT-PCR to demonstrate that in human testes VEGF mRNA was detected in Leydig cells and Sertoli cells.

Using immunohistochemical staining, Au and his co-workers (Laslett *et al.*, 1997) were the first to demonstrate the presence of flt-1 immunoreactivity in a population of testicular interstitial cells that took up the trypan blue dye, suggesting that they are testicular macrophages. In contrast, flk-1 immunoreactivity appeared to be associated exclusively with the Leydig cells. Both flt-1 and flk-1 have been partially cloned form rat testicular macrophages and Leydig cells respectively by Au *et al.*, (1998). Flt-1 is likely to be involved in the interaction between Leydig cells and macrophages as indicated by the ability of VEGF to induce testicular macrophage migration *in vitro* (Au *et al.*, 1998). In human testes, flt-1 mRNA was found by the technique of RT-PCR, to be expressed in testicular tissue, isolated fragments of testicular blood vessels and seminiferous tubules. In addition, flk-1 mRNA was also found in testicular tissue, isolated seminiferous tubules and testicular microvessels (Ergun *et al.*, 1997).

1.7 Aims of the present study

Currently, five different mammalian VEGFs have been identified and they show unique expression patterns, receptor specificity and function. The parental vascular endothelial growth factor, VEGF-A, was discovered more than a decade ago. The

recently discovered, VEGF related peptides (VEGF-B, PlGF, VEGF-C and VEGF-D) share structural features typical of the VEGF family, but display different biological activities because of their different specificities for the three known VEGF receptors (VEGFR-1, VEGFR-2 and VEGFR-3). Reports in the literature have indicated that VEGF-A is expressed mainly in Leydig cells, Sertoli cells and vascular smooth muscle cells of blood vessels in the testis. Moreover, VEGFR-1 has been localized to vascular endothelium and testicular macrophages, while VEGFR-2 to vascular endothelium, Leydig cells and Sertoli cells. In the view of above findings, it is worthwhile continuing to explore the expression and cellular localization of the newly discovered VEGF family members in the testicular tissues, which is being the aim of the present study. Furthermore, how the expression of VEGF family members in the testis respond to hCG stimulation, and how this may be related to the presence or the activity of Leydig cells will also be examined.

The present study is carried out using a combination of techniques, including RT-PCR, immunohistochemistry and Western immunoblotting.

2. Material and methods

2.1 Animals

Male Sprague-Dawley rats weighing between 350-450g were used. They were obtained from the Laboratory Animal Service Center of The Chinese University of Hong Kong. Animal studies were conducted in accordance with guidelines on the use of laboratory animals established by the Animal Ethics Committee of The Chinese University of Hong Kong. During the experiment, the rats were maintained in the animal holding room in the Department of Physiology, The Chinese University of Hong Kong. They were kept under a controlled environment of temperature 21 ± 2 ℃, humidity below 75%, and a 12 hour light (06:00 - 18:00) / 12 hour dark (18:00 - 06:00) cycle. The animals had free access to tap water and rat chow.

2.1.1 Depletion of Leydig cell

To investigate whether Leydig cells are the source of VEGFs and PlGF, ethane-1, 2-dimethane sulphonate (EDS, synthesized by The Department of Chemistry, The Chinese University of Hong Kong) treatment was used to selectively destroy the Leydig cells (Jackson & Jackson, 1984). EDS was dissolved in a dimethylsulphoxide (DMSO, Sigma, St. Louis, MO, USA) : water mixture (1:3 v/v) to a concentration of 75 mg/ml. Rats were injected intraperitoneally with a single dose of EDS (75 mg/kg bw) or the vehicle (DMSO : water). The rats were killed by

decapitation after three days and testes were removed and processed for RNA and protein extraction and immunohistochemical staining.

2.1.2 Suppression of Leydig cell and stimulation by hCG

Rats were anaesthetized by an intraperitoneal injection of 5% sodium pentobarbitone (1ml/kg) (Sigma, St. Louis, MO, USA), then received subcutaneous testosterone (Sigma, St. Louis, MO, USA)-filled silastic implants (Dow Corning, 602-305; i.d. 1.98mm, o.d. 3.18mm) of either 3cm or 25cm in length for eight weeks to achieve Leydig cell suppression. The two lengths of testosterone implants were chosen based on the fact that the 3cm implants would produce normal levels of serum testosterone (Robaire *et al.*, 1979) but could not provide adequate hormonal support to maintain normal spermatogenesis, while the 25cm implant would maintain near normal levels of spermatogenesis but in the presence of supraphysiological levels of circulating testosterone (Sun *et al.*, 1989; McLachlan *et al.*, 1994). Age-matched animals without bearing any subcutaneous silastic implants were used as the control. After 8 weeks of treatment, half the number of animals in the control and treatment groups received a single subcutaneous injection of 100 IU hCG (Pregnyl, Organon, Netherlands) in 0.2 ml phosphate-buffered saline (PBS) containing 0.01% bovine serum albumin (BSA) (Fraction V, protease-free; Sigma Chemicals Co., St. Louis, MO, USA) as the carrier protein while the other halves were injected with saline.

They were killed two days post-hCG (or saline) by decapitation. Details of the experimental design are shown in Fig.2.1.

2.1.3 Collection of tissue

The testis was decapsulated and the testicular subcapsular artery was removed. The tissue was then divided roughly into equal portions which were then placed separately into sterile 0.6ml microcentrifuge tubes and snap frozen in liquid nitrogen. The testis samples were stored at -80℃ until ready for RNA and protein extraction.

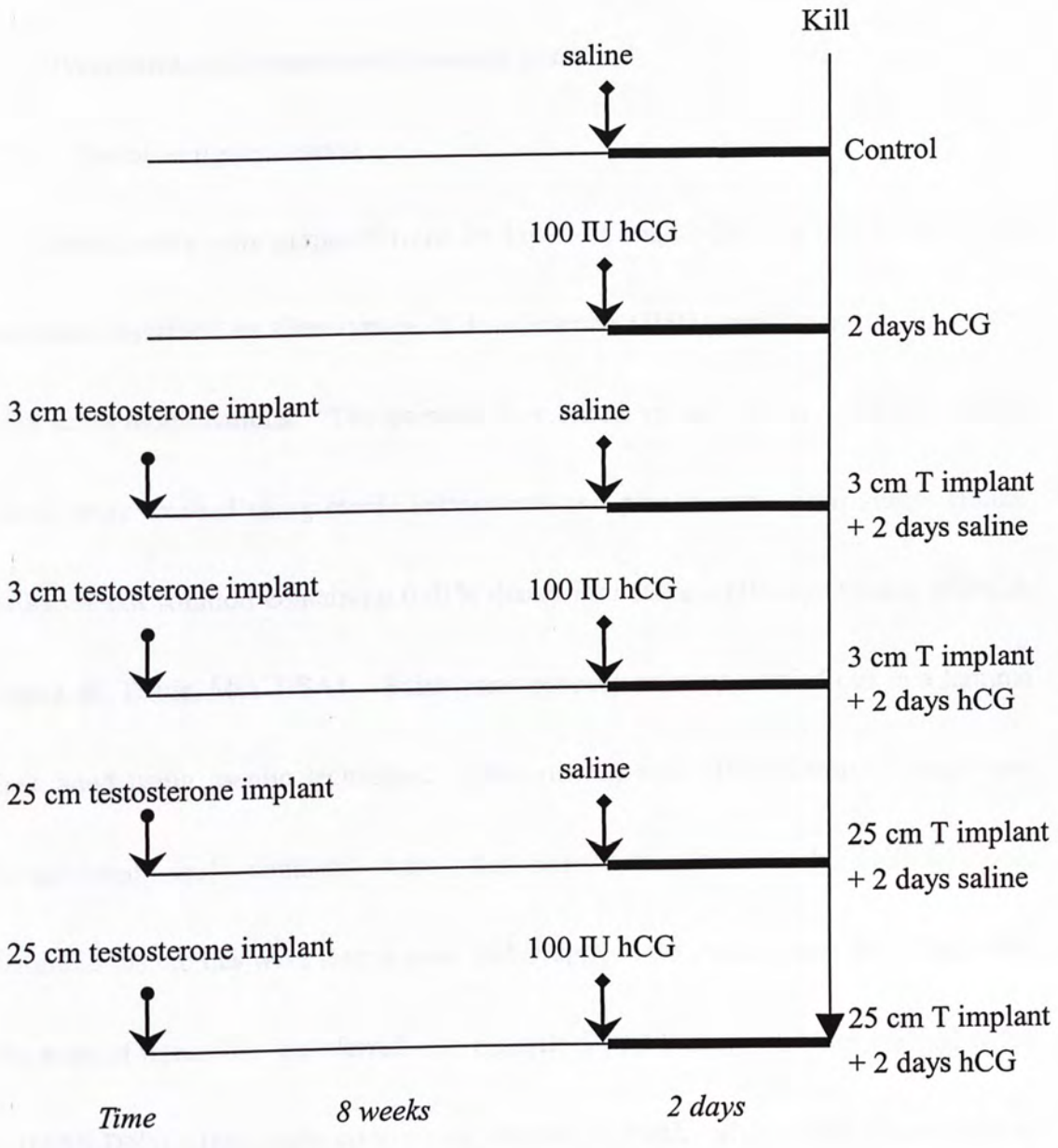


Fig.2.1 Experimental design used in the study of the effect of Leydig cell suppression by 3 cm or 25 cm testosterone (T) – filled subcutaneous silastic implants on hCG-induced endothelial cell proliferation in adult rats testes. The animals were examined at 2 days post-hCG.

2.2 Preparation of primary cells from rat testes

2.2.1 Sertoli cell preparation

Sertoli cells were prepared from 20-day-old Sprague-Dawley rats following the methods described by Gorczynska & Handelsman (1993) and Gnessi *et al.* (1995) with some modifications. The animals were killed by carbon dioxide asphyxiation. Testes were excised using sterile instruments and placed into 10 ml sterile Hanks' balanced salt solution containing 0.01% deoxyribonuclease (HBSS-DNase) (#DN25, Sigma, St. Louis, MO, USA). Subsequent procedures were carried out in a laminar flow hood using aseptic technique. After rinsing with HBSS-DNase, testes were decapsulated and contents were transferred to 5ml fresh HBSS-DNase. Seminiferous tubules were teased apart and chopped into small pieces for 15 minutes. The minced tissue was transferred into a sterile 50 ml Falcon tube and washed twice in HBSS-DNase, then made up to a total volume of 50ml. Every time after washing, the tissue was recovered by centrifugation at 250g for 3 minutes, and the supernatant was discarded. The tubule fragments were then digested in 10ml HBSS containing 0.01% DNase, 0.1% trypsin (#T8003, Sigma, St. Louis, MO, USA), 0.1% hyaluronidase (#H2551, Sigma, St. Louis, MO, USA) and 0.01% collagenase (#C9891, Sigma, St. Louis, MO, USA) in a shaking water bath (180 cycles per minute)

at 32 ℃ for 25 minutes. After digestion, the tissue was transferred to a sterile 50 ml Falcon tube and again washed twice in HBSS-DNase in a final volume of 50 ml. The supernatant was discarded each time after centrifugation at 250g for 3 min at room temperature. At the end, the tissue pellet was resuspended in 12 ml Dulbecco's Modified Eagle Medium: Ham's F12 medium (DMEM/F-12, 1:1, v/v) (Gibco BRL, Grand Island, NY, USA, Cat. No. 12500-62) supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY, USA), 5 μg/ml human transferring (#T8158, Sigma, St. Louis, MO, USA), 10μg/ml bacitracin (#BO125, Sigma, St. Louis, MO, USA), 2.5 ng/ml epidermal growth factor (#E4127, Sigma, St. Louis, MO, USA) and 10μg/ml bovine insulin (#I5500, Sigma, St. Louis, MO, USA). The cells were plated onto sterile culture plates (100 x 20 mm; Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA) and placed in a water-jacketed carbon dioxide incubator (Napco Model 5100) maintained at 32℃ under a humidified atmosphere of 5% carbon dioxide in air. The medium was changed the next day. On the second day of culture, the cultures were subjected to hypotonic treatment in 20mM TRIS (pH 7.4) for 2.5 minutes to lyses the residual germ cells. This was then followed by three washed with DMEM/F-12. On the fourth day of culture, the medium was aspirated and the cells that remained in the culture dish represented an enriched Sertoli cells preparation. They were ready for RNA and protein extraction.

It has been determined that such a Sertoli cell preparation has about 90% purity with the peritubular myoid cells as the major contaminating cell type (Ko *et al*, 2003).

2.2.2 Germ cell preparation

Germ cells were isolated from testes of 90-day-old mal Sprague-Dawley rats using the method described by Woodruff *et al.* (1992) with minor modifications. The testes were decapsulated and the tissue was transferred to a clean bottle. Twelve milliliters of DMEM/F-12 containing 30mg bovine serum albumin (#A3294, Sigma, St. Louis, MO, USA) and 3mg collagenase (Type I; Worthington Biochemical Corporation, Halls Mill Road, Freehold, New Jersey) were added and the bottle was tightly sealed. The digestion process by shaking in a water bath (110 cycles per minute) at 32℃ for 20 minutes. At the end, tubules were dispersed in 50 ml DMEM/F-12 and shaken gently. Then tissue was allowed to settle for 5 minutes and the supernatant was discarded. The tubules were further washed three times with 100 ml 10mM phosphate-buffer saline (PBS, pH 7.4, containing 9g/L sodium chloride and 1.56g/L sodium dihydrogen phosphate monohydrate) and shaken gently. Tissue was allowed to settle for 10 minutes and supernatant was discarded each time. The resulting tubules were thoroughly minced with scissors for 10 minutes and the minced tissue was transferred to a clean 50ml Falcon tube. PBS was then added to the tube to make up the final volume to 50 ml. After gentle shaking, the chucks of tissue

fragments were pelleted by centrifugation at 600g for 2 minutes. The supernatant collected after centrifugation was allowed to pass through a nylon pantyhose to remove aggregated cells and larger debris. The above steps were repeated two more times with the tissue pellets remaining at the bottom of the centrifuge tube. The resulting filtrate was added to a funnel packed with glass wool to remove the spermatozoa and then centrifuged at 100g for 4 minutes. The supernatant was discarded and the pellet at the bottom of the tube was washed twice with 50 ml PBS. At the end, the pellet was resuspended in 10ml PBS and the cell suspension was filtered through 20μm nylon mesh. The final filtrate was centrifuged at 1500g for 5 minutes. After pipetting off the supernatant, the cell pellet containing predominantly germ cells was ready for RNA and protein extraction.

2.2.3 Interstitial cell and Leydig cell preparation

The procedures used for preparing interstitial cells and Leydig cells were adopted from Murono *et al.* (1992) and Gnessi *et al.* (1995). Both cell preparations were derived from testes of 90-day-old adult Sprague-Dawley rats. Testicular interstitial cells were obtained after collagenase digestion of the testes followed by crude cell separation by centrifugation. Leydig cells were obtained after subjecting the interstitial cells to a further purification step of Percoll density gradient centrifugation.

After the animals were killed by carbon dioxide asphyxiation, their testes were removed and decapsulated. The tissue was placed in a bottle containing 24ml or 12ml 0.025% collagenase (Type I; Worthington Biochemical Corporation, Halls Mill Road, Freehold, New Jersey) solution in Medium 199 (Gibco BRL, Grand Island, NY, USA; Cat No. 21200-076) or DMEM/F-12 supplemented with 60mg BSA, 10 units/ml penicillin G sodium and 10ug/ml streptomycin sulphate (Gibco BRL, Grand Island, NY, USA) for the preparation of Leydig cells and interstitial cells, respectively. Enzyme digestion was allowed to proceed in a shaking water bath (110 cycles per minute) at 32℃ for 20 minutes. At the end, 80ml DMEM/F-12 supplemented with antibiotics and 0.2% BSA (for interstitial cell preparation) or 50 ml Medium 199 supplemented with antibiotics (for Leydig cell preparation) was added to the bottle and testicular tissue was allowed to settle for 5 minutes. The supernatant was pipetted off to 50ml Falcon tubes. For preparing Leydig cells, 25 ml medium 199 was again added to the tubes. Similarly, for preparing interstitial cells, DMEM/F-12 with 0.2% BSA was added to the tubes to make up the final volume to 50 ml. The cells were collected in the supernatant after gently swirling and until sedimentation.

After the above steps, the supernatant was pooled and centrifuged at 350g for 10 minutes. For preparing interstitial cells, 10ml PBS was added to wash the cell pellet twice. The tubes were centrifuged at 350g for 5 minutes and the supernatant was

then discarded each time. The cell pellet was finally ready for RNA and protein extraction.

For preparing Leydig cells, the cell pellet was recovered and resuspended in a small volume of Medium 199: BSA (50ml of Medium 199 supplemented with 10 units/ml penicillin G sodium, 10μg/ml streptomycin sulphate and 50mg BSA). The cells were pooled and layered on top of a discontinuous Percoll (Amersham Pharmacia Biotechnology, Buckinghamshire, UK) gradient with density of 1.030, 1.040, 1.055, 1.065, 1.070 and 1.096 g/L set up in 50ml Falcon centrifuge tube. The gradient was centrifuged at 900g for 20 minutes. Cells localizing at Percoll gradient density of 1.070g/L were saved as the Leydig cell enriched fraction. Medium 199: BSA (50ml of medium 199 supplemented with 10 units/ml penicillin G sodium and 10 μg/ml streptomycin sulphate and 50mg BSA) was added to wash the Leydig cells three times, with centrifugation at 350g for 10 minutes in between each washing step. The resulting Percoll purified Leydig cells were then ready for RNA and protein extraction.

2.3 Cell cultures

2.3.1 Reagents and cell lines

Testicular cell lines (TM3, TM4, MLTC-1, R2C and LC540) were obtained from the American Type Culture Collection (Rockville, MD) and maintained under

recommended conditions. 1:1 Dulbecco's modified Eagle Medium (DMEM): Ham's F12 Medium (Cat. No. 12500-062), RPMI1640 medium buffered with 25mM HEPES (Cat. No. 23400-021), Ham's F10 medium (Cat. No. 81200-040), Minimum Essential Medium (Eagle) in Earle's BSS (Cat. No. 41500-067), fetal bovine serum, horse serum, penicillin G (sodium salt) and streptomycin sulphate were purchased from Gibco BRL, Grand Island, New York, USA. Sodium bicarbonate and D-glucose were obtained from BDH Chemicals Ltd, Poole, England. HEPES (without sodium salt was purchased from Boehringer Mannheim, Germany. Nonpyrogenic tissue culture plates (100 x 20 mm; Polystyrene) were from Becton Dickinson Labware, Becton Dickinson and Company, Franklin Lakes, NJ, USA.

2.3.2 Mouse Leydig cell line, TM3 and Sertoli cell line, TM4

Based on information provided by the supplier (ATCC), both TM3 and TM4 cells are clonal cell lines isolated respectively from primary cultures of Leydig cell enriched preparations and Sertoli cell enriched preparations from normal testes of 11 to 13-day-old BALB/c nu/+ mice. They were maintained in a 1:1 mixture of Dulbecco's Modified Eagle Medium (DMEM)/Ham's F12 medium supplemented with 4.5g/L glucose, 1.2g/L sodium bicarbonate, 15 mM HEPES, 10 units/ml penicillin G (sodium salt) and 10μg/ml streptomycin sulphate, 5% horse serum and 2.5% fetal bovine serum. The cultures were kept at 37℃ in a water-jacketed CO_2

incubator (Nuaire, US Autoflow, Techcomp) and media were changed every 3 to 4 days. The cells were passaged at a dilution ratio of 1:10 after a period of 4 to 5 days and harvested at 80-90 % confluence.

2.3.3 Mouse tumor Leydig cell line, MLTC-1

According to the information provided by the supplier, MLTC-1 is a tumour cell line of mouse Leydig cells derived from the M548OP transplantable Leydig cell tumour carried in C57BL/6 mice. The cells were cultured in RPMI 1640 medium containing 25mM HEPES, 10% fetal bovine serum, 10 units/ml penicillin G sodium and 10μg/ml streptomycin sulphate at 37℃ under a humidified atmosphere of 5% CO_2. The medium was replaced every 3 to 4 days and the cells were passaged at a split ratio of 1:3 at 5 to 6 days intervals.

2.3.4 Rat tumor Leydig cell line, R2C

A tumour cell line of rat Leydig cells, R2C, is derived from testes of two-month-old male rat. The cells were cultured in Ham's F10 medium supplemented with 15% horse serum, 2.5% fetal bovine serum, 10 units/ml penicillin G sodium and 10μg/ml streptomycin sulphate supplements of 1.5g/L sodium bicarbonate and 2 mM L-glutamine. The cells were kept at 37℃ in a humidified 5% CO_2 atmosphere and the cells were fed every 3 days. They were passaged upon confluence at a split ratio of 1:3 at 3 to 5 days intervals.

2.3.5 Rat tumor Leydig cell line, LC540

LC540 Leydig cells are derived from Fischer male adult rat. They were grown in Minimum Essential Medium (Eagle) in Earle's BSS supplemented with non-essential amino acids, 1mM sodium pyruvate and 10% fetal bovine serum. The medium also had 10 units/ml penicillin G sodium and 10μg/ml streptomycin sulphate added as antibiotics. The cells were kept in a humidified 5% CO_2 atmosphere at 37 ℃, and the medium was changed every 3 to 4 days. The cells were passaged at a split ratio of 1:3 after a period of 3 to 5 days.

2.4 Reverse-transcription polymerase chain reaction (RT-PCR) and semi-quantitative RT-PCR

2.4.1 Extraction of total RNA

Total RNA was isolated from testicular cell lines and testicular tissues using TRIZOL reagent (1ml / 100mg) (Gibco BRL, Grand Island, NY, USA) following the procedure suggested by the manufacturer. The tissue was homogenized by hand on ice in a 3-ml glass homogenizer with a clearance of 0.004-0.006 inch (Kontes Scientific Glasswarer/ Instruments, Vineland, NJ, USA) with TRIZOL reagent. The tissue homogenate was centrifuged at 12,000g for 20 minutes at 4℃ to collect the supernatant.

For testicular cell lines and primary cells, one millilitre of TRIZOL reagent was

added to the cell pellet and the cell suspension was passed several times through a 1 ml gauge 25 syringe (Terumo, Tokyo, Japan) to disrupt the cells.

Phase separation was carried out for both tissue homogenate and cell lysate. The sample was incubated at room temperature for 15 minutes to allow thorough dissociation of nucleoprotein complexes. For every ml of TRIZOL reagent initially used for homogenization, 200 μl of chloroform was added. The sample tubes were then capped securely and hand-shaken vigorously for 15 seconds. After 3 minutes of incubation at room temperature, they were subjected to centrifugation at 11,000g for 15 minutes at 4℃. The sample mixture was separated into a lower red, phenol-chloroform phase and a colorless upper aqueous phase after centrifugation. The upper aqueous phase containing the RNA was transferred into a clean 1.5 ml microcentrifuge tube. RNA precipitation was achieved following the addition of 500 μl isopropyl alcohol to every milliliter of TRIZOL reagent initially used for homogenization, and allowing the sample to incubate at room temperature for 10 minutes. The RNA was harvested after centrifugation at 11,000g for 10 minutes at 4 ℃, washed once with 1ml 70% ethanol and then pelleted again by centrifugation at 7,500 for 5 minutes at 4℃. After carefully discarding the supernatant, the residual ethanol associated with the RNA pellet was removed by vacuum-drying for 5 to 10 minutes in the SpeedVac (DNA SpeedVac 110, Savant Instruments Inc., NY, USA).

The RNA was re-dissolved in an appropriate volume of diethylpyrocarbonate (DEPC)-treated water and stored at -70℃ until use. All the above procedures were carried out under RNase-free condition to prevent RNA degradation.

2.4.2 Quantitation of total RNA

RNA concentrations and purity were determined based on the absorbance at 260 nm (A_{260}) and 280 nm (A_{280}) by spectrophotometry (Ultrospec Spectrophotometer, Amersham Pharmacia Biotechnology, Buckinghamshire, UK) after the samples were diluted a hundredth time with DEPC-treated water. An absorbance of 1 unit at 260 nm corresponds to 40 μg of RNA per ml. The following formulae were then used to calculate RNA concentration, total yield and purity of each sample:

- RNA concentration (μg/μl) = (40 x A_{260} x dilution factor) / 1000
- Total yield (μg) = RNA concentration x total volume of RNA sample (μl)
- RNA purity = A_{260} / A_{280}

The ratio of absorbance readings taken at 260nm and 280nm (A_{260} / A_{280}) provides an estimate of the purity of the RNA sample. Only when this ratio lies between 1.5 and 1.9 would the RNA preparation be considered acceptably pure.

2.4.3 RT-PCR

Five microgram of total RNA per sample was used for cDNA synthesis employing Superscript ™ RNase H⁻ Reverse Transcriptase (200μg/ reaction) (Gibco

BRL, Grand Island, NY, USA) and oligo-deoxythymidine primers (0.5 μg/ reaction) (Gibco BRL, Grand Island, NY, USA). Reverse transcription was performed by heating 12 μl reaction mixture of 5 μg RNA sample and 0.5 μg/μl oligo-deoxythymidine primers to 70℃ for 10 minutes. Afterwards, the mixture was immediately chilled on ice for 2 minutes. Following the addition of 7 μl mixture of 5 times first strand buffer, 0.1 M dithiothreitol (DTT) and 10 mM each of the deoxynucleotidetriphosphates (dNTPs) (Gibco BRL, Grand Island, NY, USA), the reaction mixture was again heated to 42℃ for 2 minutes. 1 μl of Superscript ™ RNase H⁻ Reverse Transcriptase (200 units) was added last to the reaction mixture to reach a final volume of 20 μl. The reaction was allowed to proceed for 50 minutes at 42℃, and then followed by a final extension of 15 minutes at 72℃. The cDNA thus synthesized was stored at -20 ℃ until use. β-actin was chosen as a house-keeping gene (or as an internal control) to approximate the same amounts of cDNA used for different samples in the same PCR. The amplification of β-actin cDNA also served as an indicator of successful RNA isolation and reverse transcription.

PCR conditions for specific gene products were optimized for primer annealing temperature, concentration of magnesium chloride, PCR cycle number and initial amounts of cDNA template used for each primer set. RT-PCR experiments were

performed using pairs of specific primers for β-actin, VEGF-A, PlGF, VEGF-B, VEGF-C and VEGF-D. Primers used in the present study were purchased from Gibco BRL, Grand Island, NY, USA. Table 2.1 shows the list of primer sequences designed based on published cDNA sequences. Table 2.2 summarizes the optimal primer annealing temperature, concentration of magnesium chloride, PCR cycle number and additives used for the amplification of each PCR product. The number of cycles chosen for semi-quantitative RT-PCR was determined after a series of experiments involving template and cycle titration to establish that it was located at the exponential phase of the polymerase reaction (Fig.2.1 to 2.4).

Variable amounts of template, 10mM magnesium chloride, 2μl of 10X PCR buffer, 1μl of 10 mmoles each dATP, dGTP, dCTP and dTTP, 1 μl each of the sense and anti-sense primers (20 pmoles), and 0.5μl of 5 units per μl *Taq* DNA polymerase (Gibco BRL, Grand Island, NY, USA) were used for each PCR. It was carried out in thin wall 0.6ml PCR tubes (Robbins Scientific Corporation, Sunnyvale, CA). Master mixes were applied when possible to minimize tube-to-tube variations. The PCR amplification was performed in 20 μl volumes using the PTC-200 thermal cycler (M. J. Research, Inc., San Francisco, USA) with the program set at 94℃ for 3 minutes (initial denaturation of cDNA), 94℃ for 1 minute (optimal temperature cycles of denaturation), 58℃ for 45 seconds (primer annealing), 72℃ for 1.5 minutes

(enzymatic reaction of extension), 72℃ for 10 minutes (final extension).

The PCR products were kept at 4℃ or stored at -20℃. After PCR, 2 μl of each reaction product was analyzed by electrophoresis on 1.2 – 2.0 % agarose (Gibco BRL, Grand Island, NY, USA) gel containing 0.5 mg/ml ethidium bromide (Gibco BRL, Grand Island, NY, USA) in TAE buffer (40mM TRIS, 20mM sodium acetate, 1mM EDTA, pH 7.2). The relative intensity of the product bands were recorded and analyzed using a Fluorchem™ 8000 gel documentation system (Alpha Innotech Corporation).

For the semi-quantitation of the obtained gene signals in the treatment studies, the densitometric quantification of the gene signals were ratioed against β–actin, which was taken as an internal control, and then normalized against the control animal as 100 %.

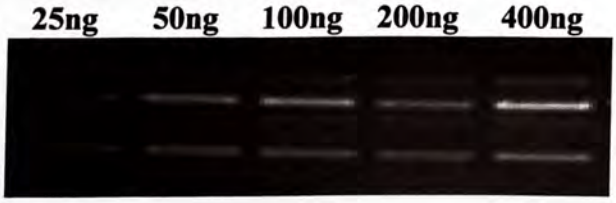
Table 2.1 Primers used for the PCR amplification of β-actin, VEGFs and VEGFRs.

Primers (S : sense; AS : antisense)		GenBank Accession Number	Primer Sequences (5' to 3')	Expected Product Size (bp)
β-actin	S	AF122902	5'-TCA CCG AGG CCC CTC TGA ACC CTA-3' (nt. 314-337)	643
	AS		5'-GGC AGT AAT CTC CTT CTG CAT CCT-3' (nt. 934-957)	
VEGF-A	S	NM_031836	5'-ATG AAC TTT CTG CTC TCT TGG GTG C-3' (nt. 1-25)	392
	AS		5'-GGT CTG CAT TCA CAT CTG CT-3' (nt. 373-392)	
VEGF-A (for spliced variants)	S	NM_031836	5'-ATG AAC TTT CTG CTC TCT TGG GTG C-3' (nt. 1-25)	645-VEGF-A_{188}, 573-VEGF-A_{164}, 513-VEGF-A_{144}, 441-VEGF-A_{120}
	AS		5'-TCA CCG CCT TGG CTT GTC ACA-3' (nt. 625-645)	
PlGF	S	NM_053595	5'-AGG TCC TAG CTG GGT TGG CTG T -3' (nt. 74-95)	383
	AS		5'-CCT CCT TTC TGC CTT TGT CGT CTC C-3' (nt. 432-456)	
VEGF-B	S	AF022952, AF032925	5'-GTG ACT GTG CAG CGC TGT GGT-3' (nt. 125-145 or 68-88)	324-$VEGF_{167}$, 425-VEGF-B_{186}
	AS		5'-GGT GTC TGG GTT GAG CTC TAA GC-3' (nt. 426-448)	
VEGF-C	S	AY032729	5'-GGA GAA AGA CTC AAT GCA TGC CAC G-3' (nt. 452-476)	253
	AS		5'-CGG CAG GAA GTG TGA TTG GCA AAA C-3' (nt. 680-704)	
VEGF-D	S	AY032728	5'-CAC TCT GAG GAC TGG AAG CTG T-3' (nt. 231-252)	233
	AS		5'-GGG GGC TTG AAG AAT GTG TTG G-3' (nt. 442-463)	

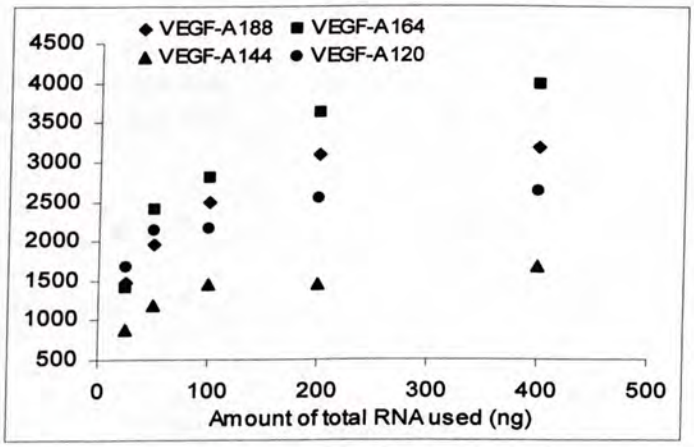
Table 2.2 Optimal primer annealing temperature, $MgCl_2$ concentration, PCR cycle numbers and additives used for each reaction.

Target	Annealing Temperature (℃)	$MgCl_2$ Concentration (mM)	PCR Cycle Numbers	Additives
β-actin	70	1.5	18	/
VEGF-A	62	1.5	28	/
VEGF-A (for spliced variants)	67	2.0	30	/
PlGF	70	1.5	30	/
VEGF-B	70	1.5	29	/
VEGF-C	70	1.5	30	/
VEGF-D	67	1.0	28	2% formamide

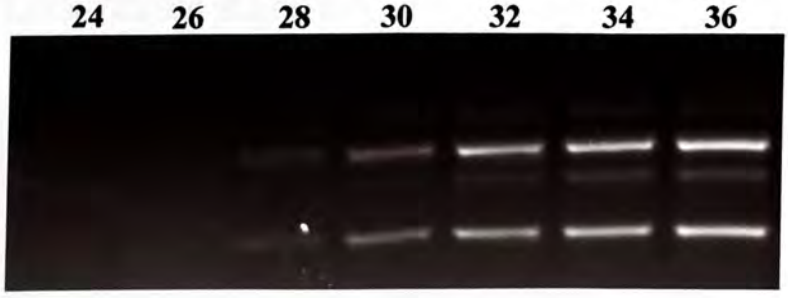
(A) **Template titration of VEGF-A (spliced variants)**



(B)



(C) **Cycle titration of VEGF-A (spliced variants)**



(D)

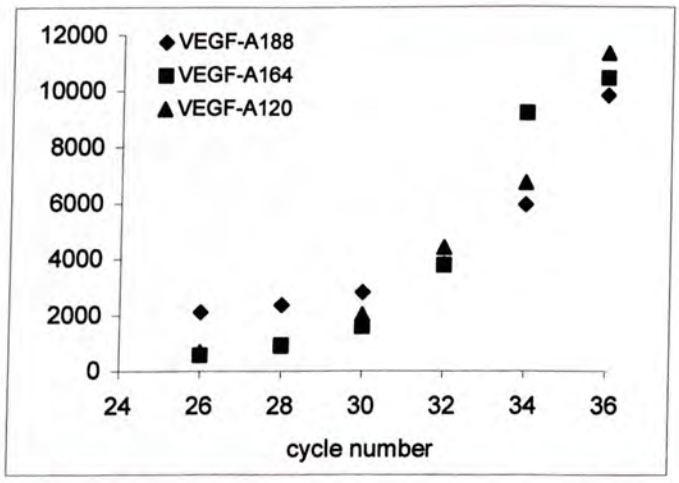
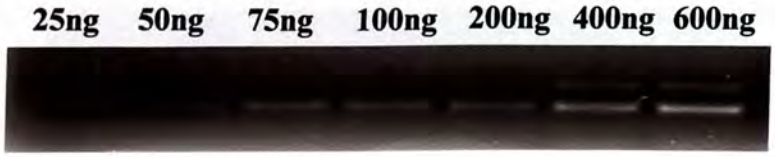
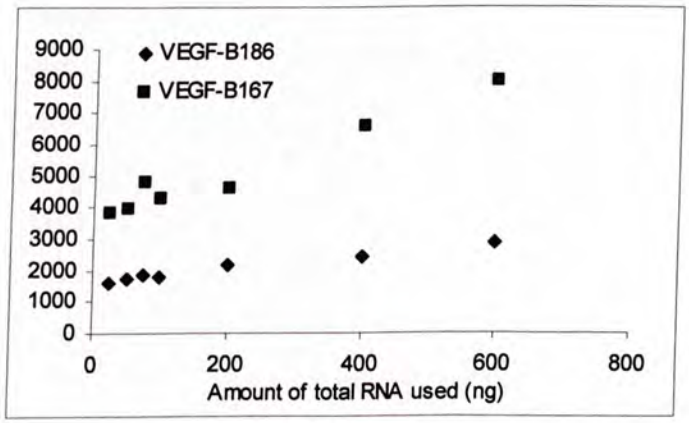


Fig.2.1 Template and cycle titration of PCR amplification for VEGF-A mRNA in the rat testis. VEGF-A gene expression in rat testis as detected by RT-PCR. **(A)-(B)** Template titration of VEGF-A mRNA, **(C)-(D)** Cycle titration of VEGF-A mRNA.

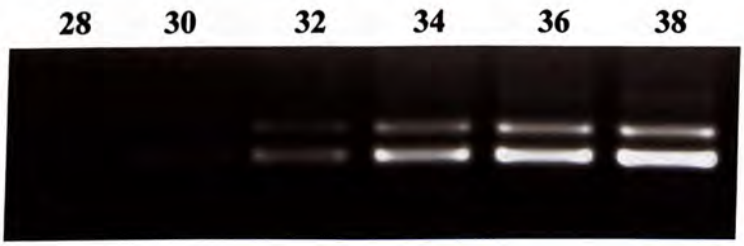
(A) **Template titration of VEGF-B**



(B)



(C) **Cycle titration of VEGF-B**



(D)

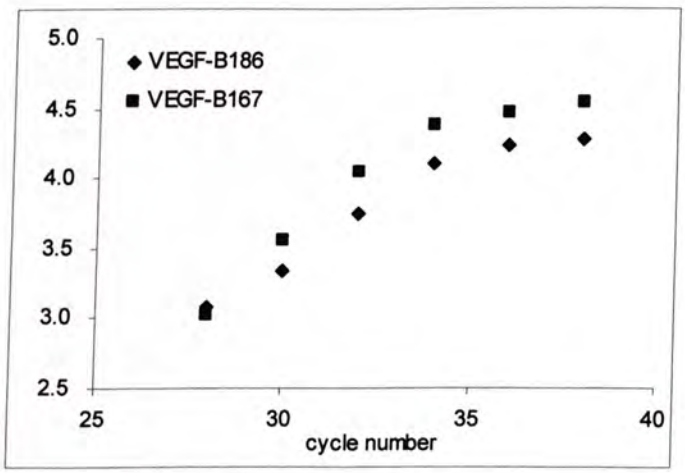
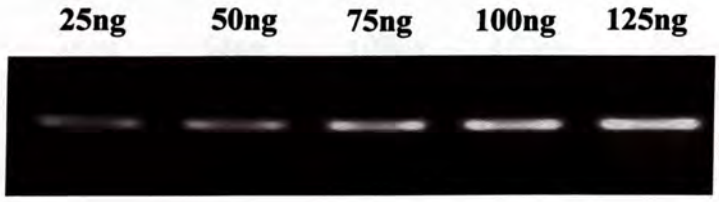
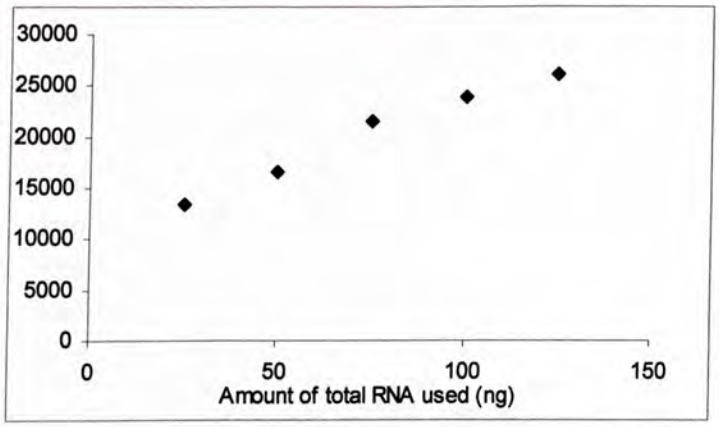


Fig.2.2 Template and cycle titration of PCR amplification for VEGF-B mRNA in the rat testis. VEGF-B gene expression in rat testis as detected by RT-PCR. **(A)-(B)** Template titration of VEGF-B mRNA, **(C)-(D)** Cycle titration of VEGF-B mRNA.

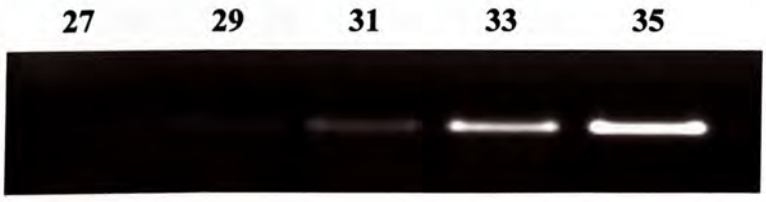
(A) **Template titration of VEGF-C**



(B)



(C) **Cycle titration of VEGF-C**



(D)

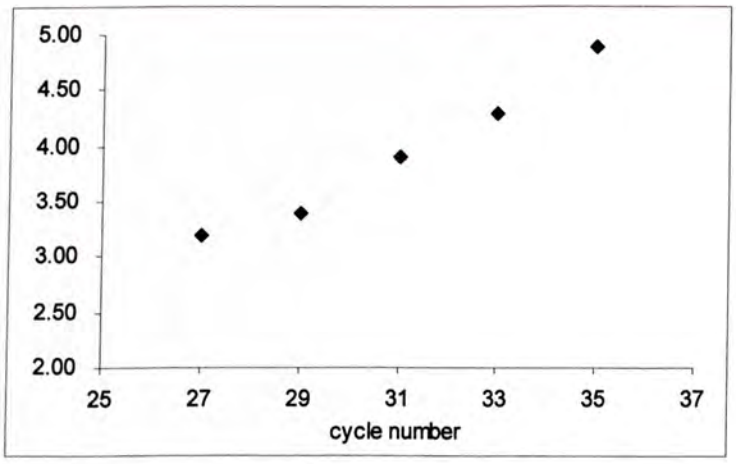
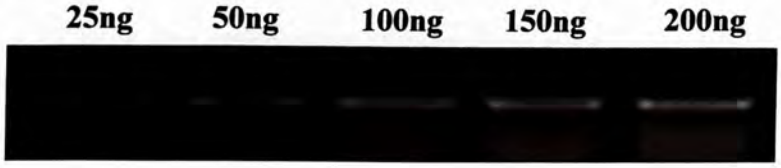
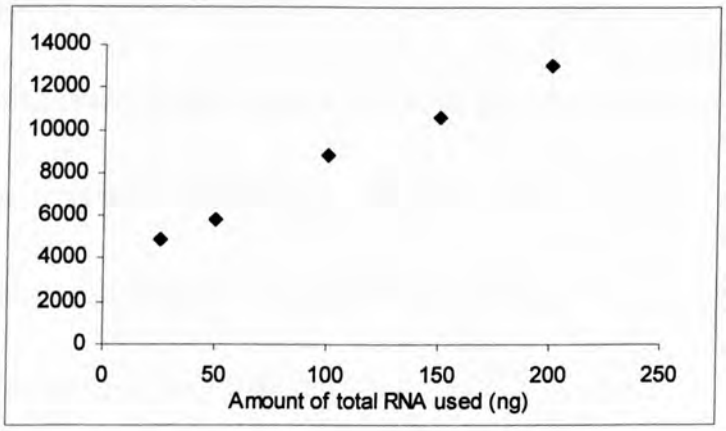


Fig.2.3 Template and cycle titration of PCR amplification for VEGF-C mRNA in the rat testis. VEGF-C gene expression in rat testis as detected by RT-PCR. **(A)-(B)** Template titration of VEGF-C mRNA, **(C)-(D)** Cycle titration of VEGF-C mRNA.

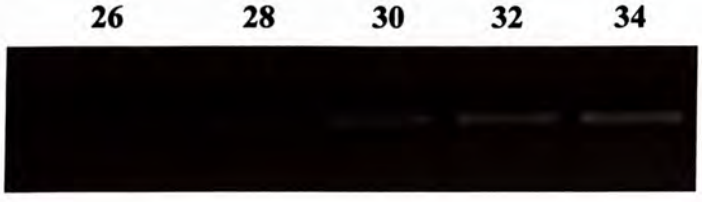
(A) **Template titration of VEGF-D**



(B)



(C) **Cycle titration of VEGF-D**



(D)

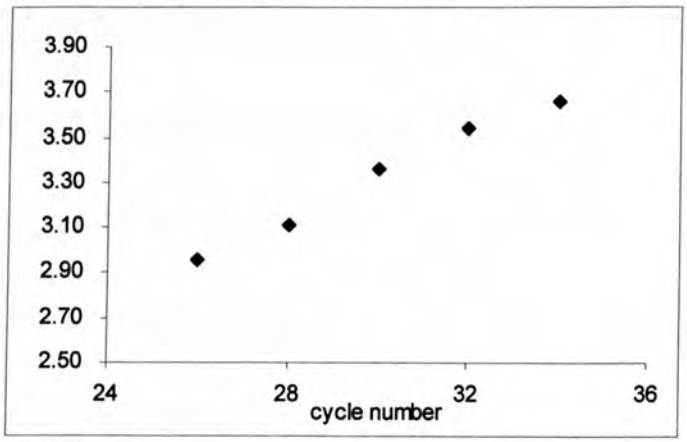


Fig.2.4 Template and cycle titration of PCR amplification for VEGF-D mRNA in the rat testis. VEGF-D gene expression in rat testis as detected by RT-PCR. **(A)-(B)** Template titration of VEGF-D mRNA, **(C)-(D)** Cycle titration of VEGF-D mRNA.

2.4.4 Purification and authentication of PCR products

Gene-Clean Kit II (Bio 101) was used to purify the amplification products. Specific bands identified after electrophoretic separation of PCR products were excised with a razor blade under transillumination using long-wave ultraviolet light. In this step, minimal amount of agarose gel was isolated and it must be carried out in the shortest time possible to avoid nicking of DNA. The net weight of the agarose gel slice was determined and used for calculating the volume of sodium iodide (6M) needed to solubilize the gel (3:1; NaI : gel) in a water bath at 55℃ with occasional mixing.

The GLASSMILK suspension was provided in the Gene-Clean Kit II, and it was thoroughly mixed by votexing before use. 5 μl of the GLASSMILK was added to each sample and incubated for 5 minutes at room temperature with occasional mixing. This GLASSMILK suspension was an aqueous suspension of proprietary silica matrix which only binds to single and double stranded DNA. Afterwards, the GLASSMILK with the bound DNA was separated by centrifugation at 11,600 g for 5 seconds. After discarding the supernatant, GLASSMILK was washed three times with 500 μl "New Wash Solution" (a concentrated solution of sodium chloride, TRIS and EDTA in water and ethanol). After the final wash and centrifugation, as much

"New Wash Solution" was removed and the pellet was resuspended in 10 μl DEPC-treated water and incubated in a water bath at 55℃ for 2 to 3 minutes to recover the DNA from the GLASSMILK. The DNA present in the supernatant was separated from the GLASSMILK after centrifugation at 11,600 g for 30 seconds. The quantity of DNA recovered was estimated by gel electrophoresis and compared against a low DNA mass ladder (Gibco BRL, Grand Island, NY, USA).

After purification, the authenticity of the PCR product was verified by forward or reverse sequencing using a dRhodamine Terminator Cycle Sequencing Kit (Perkin Elmer, Applied Biosystems, California, USA) and an automated sequencer (Advanced ABI 310 Genetic Analyzer, Perkin Elmer, Tokyo, Japan), following the procedure recommended by the supplier. The sequencing data were submitted to the BLAST Search (NCBI, National Center for Biotechnology Information; website: http://www.ncbi.nlm.nih.gov.BLAST) to confirm the authenticity of PCR products.

2.5 Immunohistochemical staining

2.5.1 Perfusion and processing of testes for histological sections

The testis was excised from the animal after it was freed from the epididymis. Using a scalpel blade (size #24, Swann-Morton, Sheffield, England), a small cut was made in the testicular subcapsular artery along its course down the epididymal margin of the testis and close to where it enters the tunica albuginea at the cranial pole of the

testis. A cannula constructed from polythene tubing (0.5mm i.d., 1.0mm o.d., 800/110/160, Portex, UK) and heat-drawn to a tip diameter of about 100μm diameter was inserted into the subcapsular artery along the direction of normal blood flow. Pre-warmed (25-30℃) heparinized saline (50 U/ml of a sodium salt of heparin from porcine intestinal mucosa in normal saline; Sigma, USA) was infused into the testis by hand using a 5ml syringe fitted with a gauge 23 needle (Terumo, Tokyo, Japan). Care was taken to prevent the entry of air bubbles. The volume of infusate (1-3 ml) was adjusted until there was a complete removal of blood. Following the saline infusion, 1.5-2.0 ml of Bouin fixative was infused until it distributed uniformly throughout the testis.

After the initial fixation by perfusion, the perfused testis was immersion-fixed in Bouin solution for 24 hours at room temperature, followed by another 24 hours at 4℃. After the first 24 hours, the testis was cut using a microtome blade (S35, Feather, Japan) into 2mm thick slices at right angle to its cranial-caudal axis.

After fixation, the testes were transferred to 70% ethanol saturated with lithium carbonate and washed 4 to 5 times to neutralize and remove picric acid left from the Bouin fixative. A slice of tissue (about 2 mm thick) was taken from the central part of each testis and put through a tissue processor for dehydration in ethanol and clearing in xylene. The timings of the above steps were as follow: 80% ethanol (2

hours), 95% ethanol (2 hours), 3 changes of 100% ethanol (1 hour each) and 2 changes of xylene (1 hour each). Then tissue sections were ready for embedding in paraplast (Paraplast X-TRA, melting point 50-54℃, Oxford Labware, St. Louis, MO, USA) after 2 changes of Paraplast X-TRA (1 1/2 hours each). Five-micron sections were cut using microtome (Model 1130, Biocut, Reichert-Jung, Germany) and mounted on glass slides coated with 1% 3-aminopropyltriethoxysilane (APES) (Sigma, St. Louis, MO, USA) in ethanol.

2.5.2 Immunohistochemical staining of tissue sections

Reagents used for immunohistochemical staining are from Zymed Laboratories Inc. (South San Francisco, USA) and they are based on the streptavidin-biotin-peroxidase method. The procedures were carried out according to the manufacturer's instructions with some modifications. Five microns testis sections were dewaxed in two changes of (R)-(+)-limonen (Merck, Germany) (4 minutes each), rehydrated through a down-series of ethanol (2 changes of 2 minutes each in absolute ethanol (Merck, Germany), one wash for 2 minutes in 95% ethanol, one wash for 5 minutes in 70% ethanol saturated with lithium carbonate), then placed for 5 minutes in distilled water, 5 minutes in 300mM glycine (Bio-Rad, CA, USA) and finally equilibrated in 10mM phosphate-buffer saline (PBS, pH 7.4). The tissue endogenous peroxidase activity was quenched by treatment with 3% H_2O_2 (Merck,

Germany) in PBS for 10 minutes.

Before reacting with the antibodies, the testis sections were further subjected to an antigen retrieval procedure to unmask the antigenic sites for antibody binding. The slides were heated in 0.01M citrate buffer (pH 4.1) for 20 minutes at 95℃ and then left to cool in the same buffer for 20 minutes at room temperature.

The VEGF antibodies, including VEGF-A (A20), VEGF-B (P19), VEGF-C (H190) and VEGF-D (H144) were commercially available from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Owing to no suitable commercially available PlGF antibody could be used for the immunohistochemical staining, this part of study was omitted. They were used at 1:200 for VEGF-A and 1:50 for the others in PBS containing 20% fetal calf serum (FCS) (Gibco BRL, Grand Island, NY, USA). The incubation was carried out for one hour at room temperature, after which the antibody was rinsed off and replaced by the biotinylated secondary antibody (goat anti-rabbit IgG for VEGF-A, C, D or swine anti-goat IgG for VEGF-B) used at 1:75 dilution for 10 minutes at room temperature. In between steps, the sections were rinsed three times at 2 minutes intervals with PBS. Following reaction with the second antibody, peroxidase-conjugated streptavidin (diluted 1:400 in PBS) was added and left for 10 minutes at room temperature. Finally, specific staining of the antigen was visualized by the addition of the chromogen, the AEC reagent, which gave a red color after

15-30 minutes incubation at room temperature. After immunostaining, the sections were counterstained with haematoxylin and mounted without dehydration in an aqueous mountant (polyvinyl pyrrolidone).

The positive immunostaining was examined under light microscope and areas of interest were recorded using a Leica microscope fitted with a high resolution CCD camera. The images were captured on computer files using the LEICA QWin Standard (version. 2.0 for Windows, Leica Imaging System).

2.6 Western immunoblotting

2.6.1 Extraction and quantitation of total protein

Tissues were homogenized by hand on ice in 3 ml glass homogenizers with clearance of 0.004-0.006 inch (Kontes Scientific Glasswarer/ Instruments, Vineland, NJ, USA) and cell pellets were passed several times through a 1 ml gauge 25 syringe (Terumo, Tokyo, Japan) to disrupt the cells in 10 mM Tris-HCl buffer (pH 7.4) supplemented with protease inhibitors (1μg/ml leupetin, 5μg/ml aprotonin, 100μg/ml phenylmethylsulfonyl fluoride (PMSF), 1mM sodium orthovanadate, 1mM EGTA, 1mM EDTA, and phosphatase inhibitors containing sodium fluoride, β-glycerolphosphate and HEPES at pH 7.3). The supernatant was collected after centrifugation at 15,000g for 20 minutes at 4℃. All procedures were done on ice or at 4℃ to minimize protein degradation. The protein concentration of tissue

homogenates was determined using the Bio-Rad DC Protein Assay (Bio-Rad, CA, USA) according to the manufacturer's instructions. After determination of the protein concentration, the samples were aliquoted and stored at -80℃ until use.

Each tissue lysate was mixed with an equal volume of double-strength electrophoresis sample buffer (125 mM Tris, pH 6.8, 4% sodium dodecyl-sulphate (SDS), 20% glycerol, 10% β-mercaptoethanol) and boiled for 5 minutes at 100℃ to denature the protein. For the immunoblotting of VEGFs and its receptors, 40 μg and 80 μg of total protein were used respectively for each sample.

2.6.2 SDS-PAGE

15% acrylamide gel (Bio-Rad, CA, USA) was used for the separation of VEGFs and PlGF. They were prepared by mixing deionized water (1 volume for 15%, 2 volumes for 7.5%), Tris-base buffer (pH 8.8, Bio-Rad, CA, USA) (1 volume for 15% and 7.5%) and acrylamide: bisacrylamide (Bio-Rad, CA, USA) (37.5:1 w/w) (30% w/v solution in H_2O) (2 volumes for 15%, 1 volume for 7%). Tetramethylethylene diamide (TEMED, Bio-Rad, CA, USA) and freshly prepared ammonium persulphate (Bio-Rad, CA, USA) were then added to a final concentration of 0.1%. After thorough mixing, it was transferred to fill the space between the two glass plates on the casting stand. Isopropanol was overlay on top of the gel to give it a level surface, and the gel was allowed to polymerize for 40 minutes. After the separating gel had

set, isopropanol was discarded and the top of the gel was rinsed five times with deionized water. The area above the separating gel was dried by soaking with filter paper before the stacking gel was poured.

4% acrylamide stacking gel was prepared by thoroughly mixing water (4.5 volumes), upper Tris-base buffer (pH 6.8) (2 volumes), acrylamide: bisacrylamide (37.5:1 w/w) (30% w/v solution in H_2O) (1 volume), tetramethylethylene diamide (TEMED, to a final concentration of 2%) and freshly prepared ammonium persulphate (to a final concentration of 0.1%). Then it was overlay on top of the separating gel, and the comb used for casting the sample wells was inserted. The polymerization was allowed to proceed for 40 minutes.

After the gel had set, the comb was carefully withdrawn and the wells were rinsed 3 times with Tris-glycine electrophoresis running buffer. The wells were kept moist by filling them with running buffer, and the sandwich unit of gel and glass plates was clamped to the electrophoresis assembly.

Denatured protein samples (40 or 80 μg per lane), prestained molecular size markers (Low range molecular size prestained marker showing band sizes of 21.4 to 111 kDa, Bio-Rad, CA, USA) and a biotinylated molecular size marker (showing band sizes of 14.3 – 97.4kDa, Sigma, St. Louis, MO, USA) were loaded respectively into each sample well of the polyacrylamide gel. After placing the electrophoresis

assembly unit into the lower buffer chamber, both the upper and lower chambers were filled with running buffer. Air bubbles adhering to the lower edge of the gel were removed using a U-shaped pasteur pipette. After checking for leakage, the electrophoresis unit was connected to a power supply. Electrophoresis was performed under a constant voltage of 60 – 120 V until the proteins had achieved a desirable separation as indicated by the migration of the prestained marker.

2.6.3 Immunoblotting

The polyacrylamide gel was removed from the electrophoresis assembly and equilibrated in transfer buffer (Tris-glycine, 0.0375% SDS, 20% methanol) for 20 minutes. Polyvinylidene difluoride membrane (PVDF, Immobilon-PSQ, Millipore Corporation, Bedford, USA) was soaked briefly in methanol for 15 seconds and then in transfer buffer for another 20 minutes.

For VEGFs and PlGF, electroblotting was carried out either using a Trans-Blot Semi-Dry Electrophoretic Transfer Cell (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions for 35 minutes under a constant voltage of 18V, or wet transfer (which was performed at about 4℃ by placing an ice block inside the transfer tank) according to the manufacturer's instructions in a Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories, Hercules, CA, USA) for 1 hour at a constant voltage of 100V.

After electroblotting, the membrane was briefly washed three times with deionized water before being blocked for one hour at room temperature with 5% skim milk (Carnation) for VEGFs or 2% bovine serum albumin (BSA, Sigma, St. Louis, MO, USA) for PlGF. The blocking solutions were made up in PBS-Tween (polyoxyethylene sorbitan monolaurate, Sigma, St. Louis, MO, USA) (PBST). Overnight incubation with rabbit polyclonal primary antibodies [1:500 dilution for VEGF-A (A-20), VEGF-B (H70), VEGF-C (H190) and VEGF-D (H144) in 5% skim milk, and PlGF (R18) in 2% BSA at room temperature] were carried out on a roller mixer. On the next day, membrane was then washed three times with PBST and incubated with secondary peroxidase-conjugated goat-anti-rabbit antibody or secondary peroxidase-conjugated rabbit-anti-goat antibody (DAKO A/S, Denmark) (1:3000 or 1:5000 dilution in blocking reagent) for 1 hour in room temperature. After further washing, specific protein bands on the membrane were visualized after incubation in ECL reagent (Amersham Pharmacia Biotechnology, Buckinghamshire, UK) for 1 minute followed by exposure to X-ray film (FujiFilm, Japan). The position of the biotinylated molecular size markers were subsequently determined after incubating the membrane with streptavidin-peroxidase (1:5000 dilution) for 30 minutes at room temperature followed by chemiluminescence detection using ECL reagent and X-ray film. In the control experiment, incubation with the primary

antibody was omitted and the blot was only exposed to the diluent of the secondary antibodies. This served to determine if any of the signals that finally appeared would be non-specific.

2.7 Statistical analyses

Data were expressed as mean ± standard error, with the number of animals or replicates given by the value of n. Statistical analyses were performed using a commercially available software package (SigmaStat for Windows, ver. 1.0, Jandel Scientific Software, San Rafael, CA, USA). Non-parametric tests were used for data that were expressed as percentages of the control. Kruskal-Wallis one-way ANOVA on Ranks was used to determine if significant differences existed among the various experimental groups. This was then followed by the use of Dunnett's test for comparing treatment groups against the control. For comparison between two groups, Mann-Whitney rank sum test was used. P value of less than 0.05 was taken as statistically significant.

3. Results

3.1 Expression and localization of VEGFs in the rat testis

3.1.1 VEGF-A

Using immunohistochemical staining, VEGF-A immunoreactivity was localized mainly in the intertubular area within the cytoplasm of the interstitial cells which were mainly composed of Leydig cells. Moreover, immunoreactivity was found in Sertoli cells and vascular smooth muscle cells, though their immunoreactivity appeared weaker (Fig.3.1A and B).

In this and subsequent immunostaining studies, the specificity of the immunohistochemical staining was tested by omitting of the primary antibody. They all resulted in the complete disappearance of the positive immunoreactivity with no background staining (Fig 3.1 I to L).

In the RT-PCR analysis, VEGF-A transcripts were demonstrated in the normal rat testis, cultured rat primary cells and cell lines by specific primers, though they showed different expression patterns and intensities (Fig.3.2). PCR products separated on ethidium bromide-stained agarose gel showed four mRNA spliced variants of with expected sizes of 645 bp, 573 bp, 513 bp and 441 bp encoding the four VEGF-A isoforms of VEGF-A_{188}, VEGF-A_{164}, VEGF-A_{144} and VEGF-A_{120} respectively. Among the four isoforms, VEGF-A_{188}, VEGF-A_{164} and VEGF-A_{120} appeared to be the major forms and were found in most cell types, including Leydig cell and Sertoli cell. The less abundant VEGF-A_{144} transcript was clearly demonstrated in total RNA extracted from cells but not testicular tissue. The five testicular cell lines cover two cell types: Leydig cells (LC540, R2C, MLTC-1 and TM3) and Sertoli cells (TM4). According to this categorization, VEGF-A transcripts found in all these cell lines was an evidence

confirming that VEGF-A transcripts were expressed in Leydig cells and Sertoli cells (Fig.3.2B).

By adjusting the amounts of template used in PCR based on the signal intensity from the amplification of the internal control, β-actin, and the PCR was conducted during the exponential phase of the reaction, a semi-quantitative estimate of the relative abundance of VEGF-A mRNA in different cell types was achieved. Based on this semi-quantitative comparison, VEGF-A transcript expression in the Leydig cell appeared to exhibit the strongest signal among the primary cells (Fig.3.2A), indicating that it probably was the major source of VEGF-A transcripts in the testis.

The authenticity of the products was confirmed by DNA sequencing using an automated DNA sequencing and analysis system (data not included). Primers were designed in such a way that they span over a region of intron of the targeted gene such that any genomic DNA contamination in the RNA and hence cDNA preparation, would yield PCR products of larger sizes. Furthermore, this was regularly tested by showing the absence of signals from RT-PCR performed without the addition of reverse transcriptase (data not shown).

Using a specific antibody, Western blot analysis illustrated three major protein bands with apparent molecular mass of 27 kDa, 21 kDa and 15 kDa. Since the electrophoretic separation was carried out under reducing conditions, these three protein bands corresponded to the reported sizes of the monomers of VEGF-A_{188}, VEGF-A_{164} and VEGF-A_{120} respectively. They were present in cultured primary cells, cell lines and rat testis with different abundance and expression patterns (Fig.3.3 and 3.4). In line with the findings from semi-quantitative analysis of the relative abundance of VEGF-A transcripts in different primary cell types together with adjustment of loading based on the same amount of total protein, VEGF-A peptide levels appeared to be the highest in

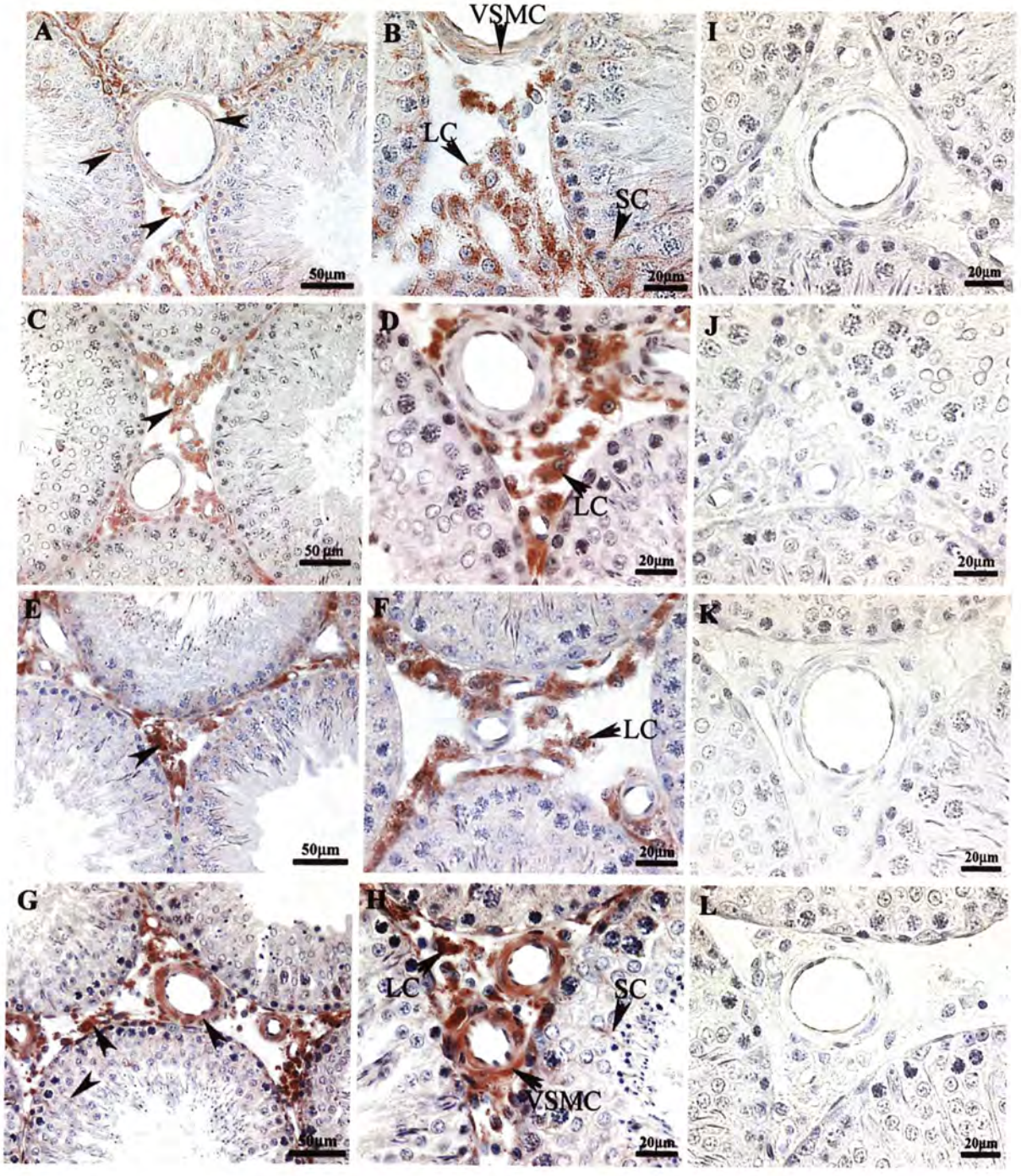


Fig.3.1 Immunohistochemical staining of VEGFs in the normal adult rat testes. Positive VEGFs immunoreactivity was indicated by the red colour and arrows in tissue sections that were counterstained with haematoxylin. In (**A-B**), immunoreactivity of VEGF-A was localized in the cytoplasm of Leydig cells (LC), Sertoli cells (SC) and vascular smooth muscle cells (VSMC). In (**C-D**), immunoreactivity of VEGF-B was concentrated in the Leydig cells, which was similar to the immunoreactivity of VEGF-C observed in (**E-F**). In (**G-H**), immunoreactivity of VEGF-D was mainly localized in the Leydig cells and vascular smooth muscle cells, but weaker in the Sertoli cells. (**I-L**) were the negative controls of immunostaining study of VEGF-A, B, C and D respectively, prepared by omitting the primary antibody in the staining processes.

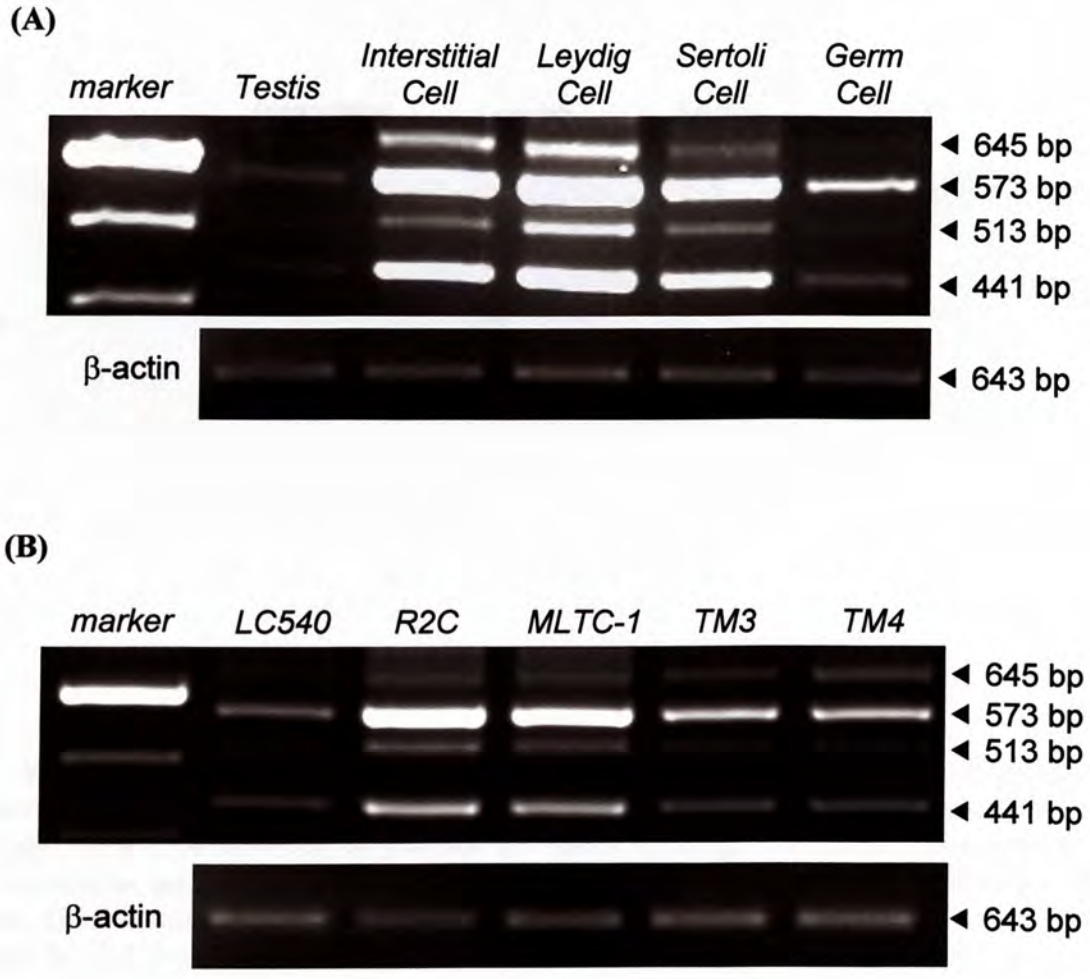


Fig.3.2 RT-PCR analysis of VEGF-A mRNA expression in rat testis, testicular primary cells (A) and testicular cell lines (B). Four major spliced variants of VEGF-A mRNA (645bp, 573bp, 513bp and 441bp) representing VEGF-A_{188}, VEGF-A_{164}, VEGF-A_{144} and VEGF-A_{120} respectively were found in all samples with weaker signals in the testis and germ cells. The amount of template used in PCR was adjusted based on prior amplification of an internal control, β-actin, with a product size of 643bp (lower panel) which gave similar signal intensities for each samples.

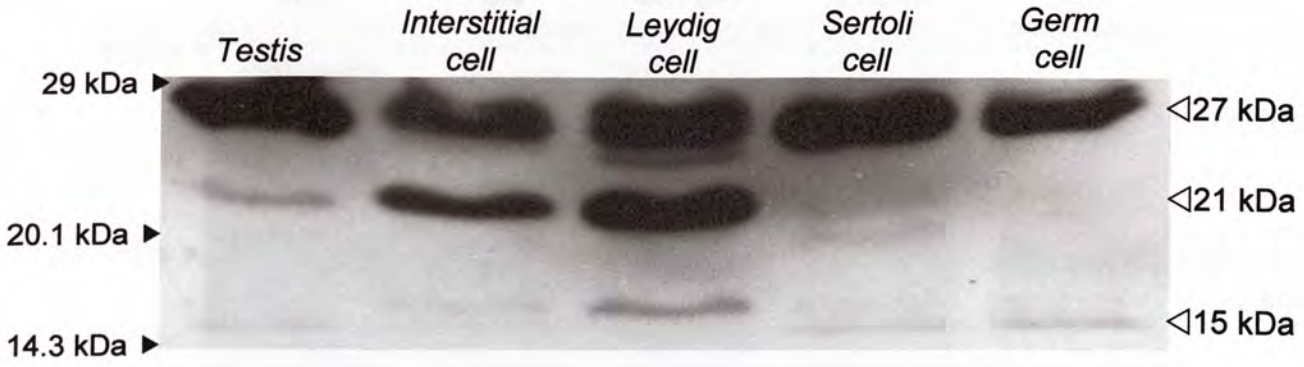


Fig.3.3 Western blot analysis of VEGF-A protein expression in rat testis and primary testicular cells. Equal amount of total protein from each tissue or cell lysate was size fractionated on a 15% SDS-polyacrylamide gel under reducing conditions, transferred to a PVDF membrane, and immunoblotted with a rabbit polyclonal anti-VEGF-A antibody. The blot was then incubated with horseradish peroxidase-conjugated anti-rabbit IgG and visualized by the ECL detection system. The antibody recognized proteins with apparent molecular mass of 27 kDa, 21 kDa and 15 kDa representing different isoforms of VEGF-A, namely VEGF-A_{188}, VEGF-A_{164} and VEGF-A_{120} respectively. The positions of molecular mass markers were shown on the left.

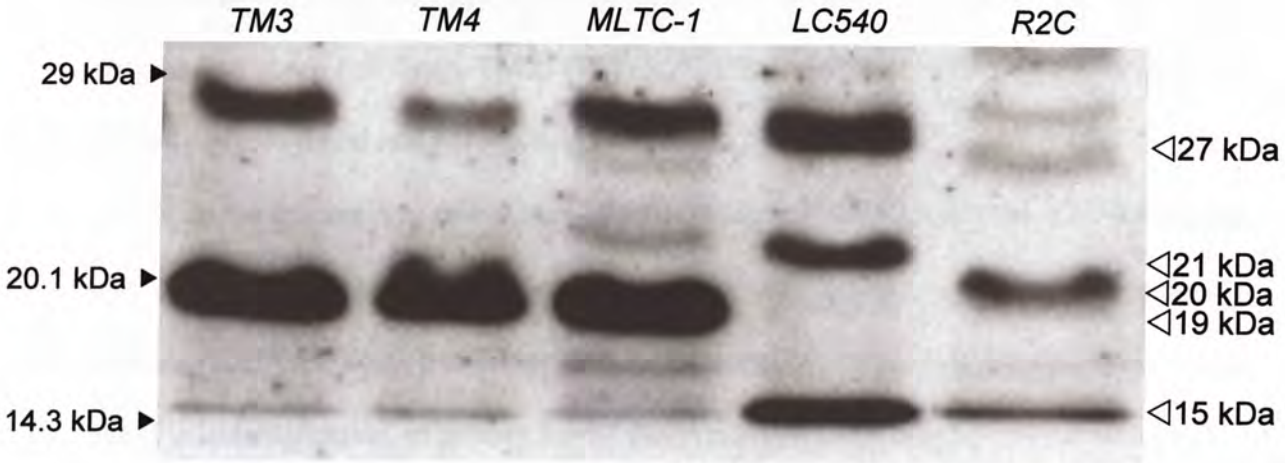


Fig.3.4 Western blot analysis of VEGF-A protein expression in various testicular cell lines. Equal amount of total protein from cell lysate of each testicular cell line was size fractionated on a 15% SDS-polyacrylamide gel under reducing conditions, transferred to a PVDF membrane, and immunoblotted with a rabbit polyclonal anti-VEGF-A antibody. The blot was then incubated with horseradish peroxidase-conjugated anti-rabbit IgG and visualized by the ECL detection system. The antibody recognized proteins with apparent molecular mass of 27 kDa, 21 kDa and 15 kDa representing different isoforms of VEGF-A, namely VEGF-A_{188}, VEGF-A_{164} and VEGF-A_{120} respectively. The bands of molecular mass of 20 kDa and 19 kDa appeared on cell lines, TM3, TM4, MLTC-1 and R2C might represent the native protein that is partially or not glycosylated since the calculated molecular mass of $VEGF_{164}$ is 19 kDa. The positions of molecular mass markers were shown on the left.

the Leydig cell (Fig.3.3). Among the three isoforms, VEGF-A_{188} was the most abundant in testicular tissues and primary cells (Fig.3.3) while VEGF-A_{164} appeared more abundant in cell lines. In Fig.3.4, protein bands of molecular mass of 20 kDa and 19 kDa appeared in some cell lines, TM3, TM4, MLTC-1 and R2C. They might represent the partially glycosylated and non-glycosylated forms of VEGF-A_{164}.

In the present and subsequent Western blot analyses, the specificity of the signals was confirmed by omitting the incubation with primary or secondary antibody and demonstrated their complete disappearance from the blot (data not shown). This would avoid misidentification of protein bands that were caused by non-specific binding to the primary and secondary antibodies.

3.1.2 VEGF-B

In immunohistochemical study, VEGF-B immunoreactivity was localized in the interstitium where the Leydig cells were located (Fig.3.1C and D). No immunoreactivity was found in Sertoli cells or germ cells as would be expected from the RT-PCR and Western blot results. This might be explained by the fact that not all the epitopes were retrieved and exposed to specific antibody during the immunostaining process.

Using specific primers, VEGF-B transcripts were demonstrated in all cultured cell types and rat testis in the RT-PCR analysis, and they showed similar expression patterns and intensities (Fig.3.5). Two products were found and they corresponded with size to those expected from the amplification of VEGF-B_{186} and VEGF-B_{167} transcripts (i.e. 425 bp and 324 bp). RT-PCR results showed that VEGF-B transcripts were expressed in Leydig cells and Sertoli cell.

Western blot analysis showed two major protein bands with apparent molecular mass of 35 kDa and 24 kDa in cultured primary cells, cell lines and testis with different

abundance and expression patterns (Fig.3.6 and 3.7). Under reducing conditions, these two protein bands corresponded in size to those reported for the glycosylated full length form of VEGF-B_{186} and VEGF-B_{167}, respectively. The other protein bands with apparent molecular mass of 30 kDa and 19 kDa were probably the proteolytically processed forms of the VEGF-B_{186} and VEGF-B_{167}. Based on the Western blot results, VEGF-B_{186} peptide (after adjustment of loading based on the same amount of total protein) appeared to be the most abundant in the Leydig cell (Fig.3.6).

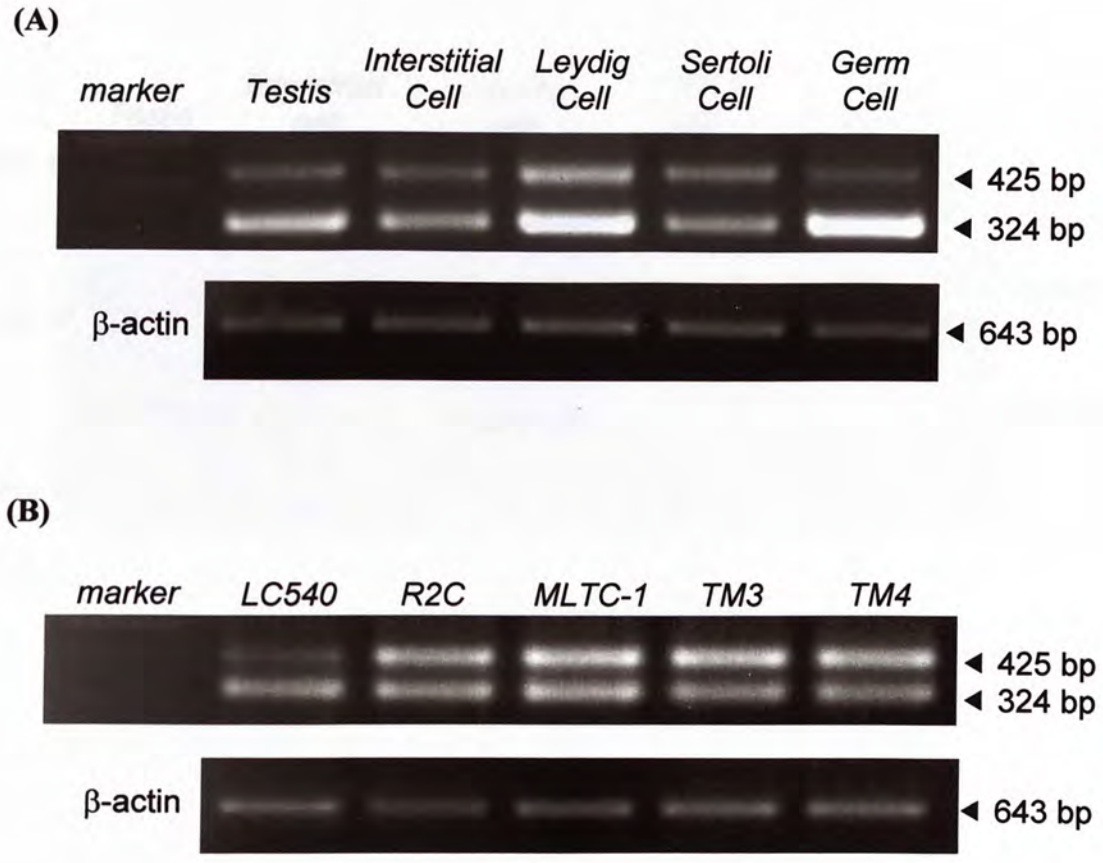


Fig.3.5 RT-PCR analysis of VEGF-B mRNA expression in rat testis, testicular primary cells (A) and testicular cell lines (B). Two PCR products corresponding with size to what would be expected (i.e. 425 bp and 324 bp) from the amplification of VEGF-B_{186} and VEGF-B_{167} transcripts respectively were obtained. The amount of template used in PCR was adjusted based on prior amplification of an internal control, β-actin, with a product size of 643bp (lower panel) which gave similar signal intensities for each samples.

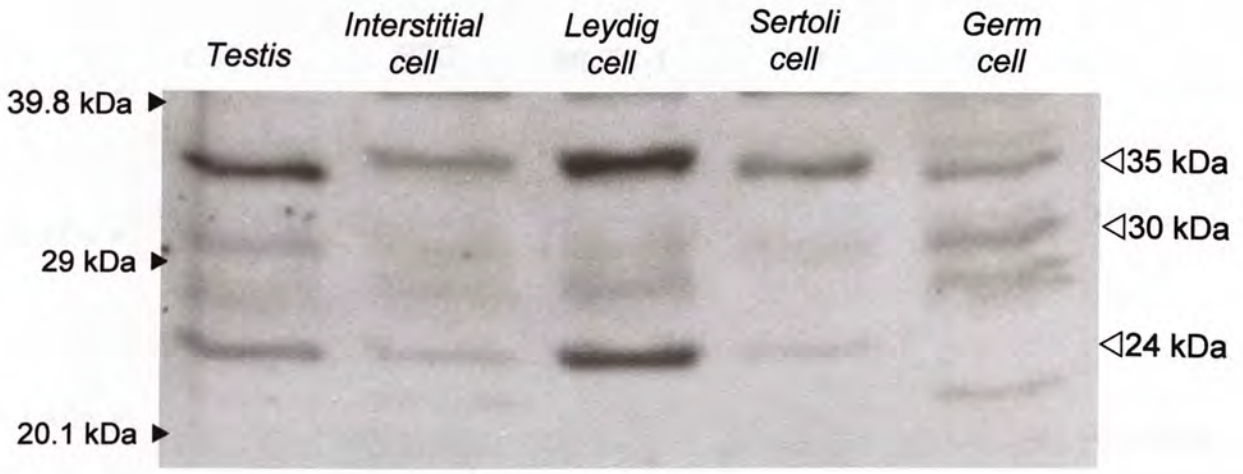


Fig.3.6 Western blot analysis of VEGF-B protein expression in rat testis and primary testicular cells. Equal amount of total protein from each tissue or cell lysate of was size fractionated on a 15% SDS-polyacrylamide gel under reducing conditions, transferred to a PVDF membrane, and immunoblotted with a rabbit polyclonal anti-VEGF-B antibody. The blot was then incubated with horseradish peroxidase-conjugated anti-rabbit IgG and visualized by the ECL detection system. The antibody recognized proteins with apparent molecular mass of 35 kDa, 30 kDa and 24 kDa representing different isoforms of VEGF-B. The positions of molecular mass markers were shown on the left.

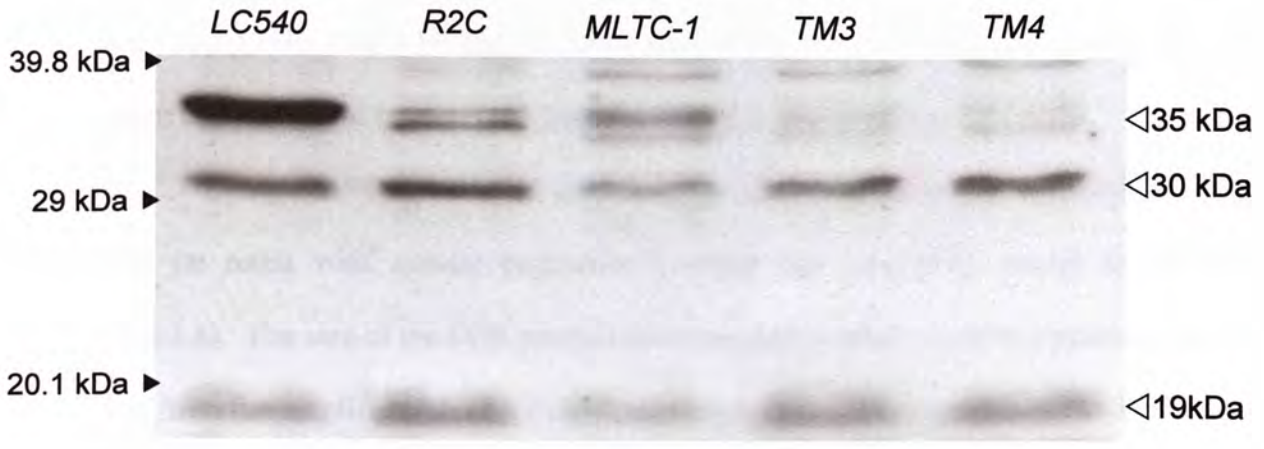


Fig.3.7 Western blot analysis of VEGF-B protein expression in various testicular cell lines. Equal amount of total protein from cell lysate of each testicular cell line was size fractionated on a 15% SDS-polyacrylamide gel under reducing conditions, transferred to a PVDF membrane, and immunoblotted with a rabbit polyclonal anti-VEGF-B antibody. The blot was then incubated with horseradish peroxidase-conjugated anti-rabbit IgG and visualized by the ECL detection system. The antibody recognized proteins with apparent molecular mass of 35 kDa, 30 kDa and 19 kDa representing different isoforms of VEGF-B. The positions of molecular mass markers were shown on the left.

3.1.3 VEGF-C

Immunohistochemical staining illustrated that VEGF-C was chiefly localized in the Leydig cells (Fig.3.1E and F). Little immunoreactivity was found in Sertoli cells and germ cells as would be expected from the RT-PCR and Western blot results.

Using RT-PCR, VEGF-C mRNA appeared to be present in all cultured cell types and rat testis with similar expression patterns and intensities, except in MLTC-1 (Fig.3.8). The size of the PCR product corresponded to what would be expected (i.e. 253 bp) from the amplification of VEGF-C transcript. Results demonstrated that Leydig cell was the major source of VEGF-C transcripts.

Western blot analysis showed one major protein band with apparent molecular mass of 27 kDa in cultured primary cells, cell lines (except LC540) and testis with very different abundance and expression patterns (Fig.3.9 and 3.10). Under reducing conditions, this protein band might correspond to the proteolytically processed form of VEGF-C. The other protein band with apparent molecular mass of 25 kDa was probably a variant of the processed form of VEGF-C peptide. From Western blot (Fig.3.9); VEGF-C peptide (after adjustment of loading based on the same amount of total protein) appeared to be the most abundant in the Leydig cells, and less in the Sertoli cells.

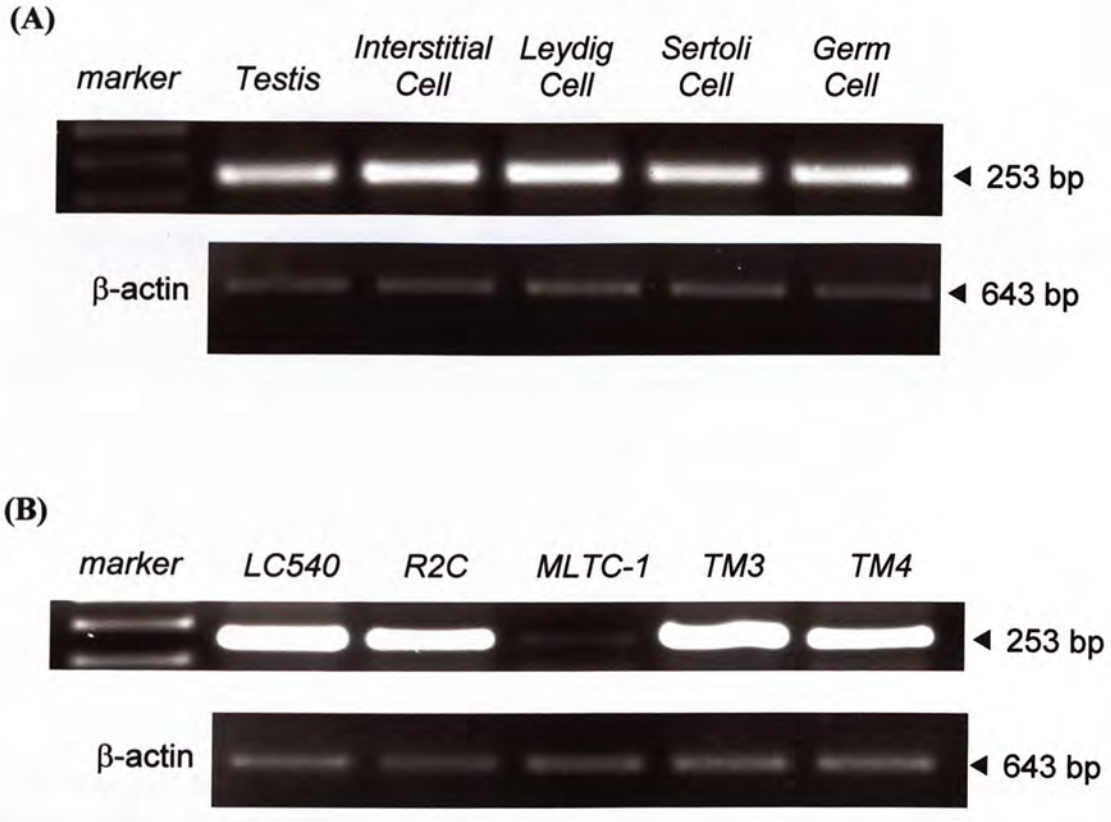


Fig.3.8 RT-PCR analysis of VEGF-C mRNA expression in rat testis, testicular primary cells (A) and testicular cell lines (B). A PCR product corresponding in size to what would be expected (i.e. 253 bp) from the amplification of VEGF-C transcript was found, with a weaker signal seen in MLTC-1 cells. The amount of template used in PCR was adjusted based on prior amplification of an internal control, β-actin, with a product size of 643bp (lower panel) which gave similar signal intensities for each samples.

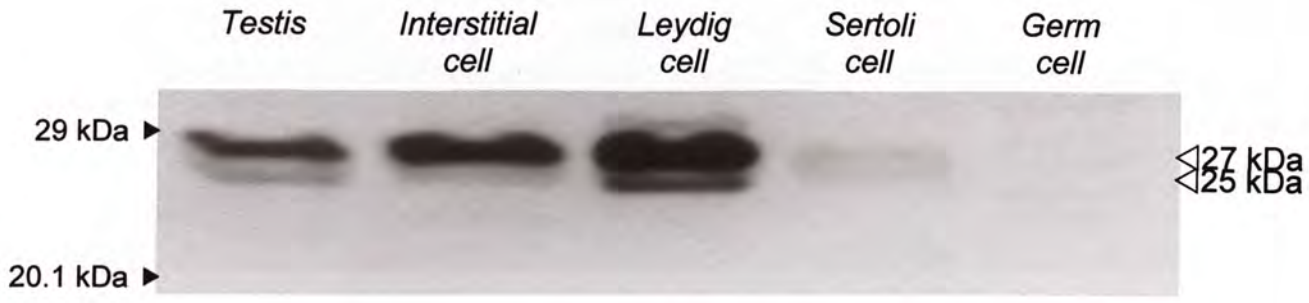


Fig.3.9 Western blot analysis of VEGF-C protein expression in rat testis and primary testicular cells. Equal amount of total protein from each tissue or cell lysate was size fractionated on a 12.5% SDS-polyacrylamide gel under reducing conditions, transferred to a PVDF membrane, and immunoblotted with a rabbit polyclonal anti-VEGF-C antibody. The blot was then incubated with horseradish peroxidase-conjugated anti-rabbit IgG and visualized by the ECL detection system. The antibody recognized proteins with apparent molecular mass of 27 kDa and 25 kDa. The positions of molecular mass markers were shown on the left.

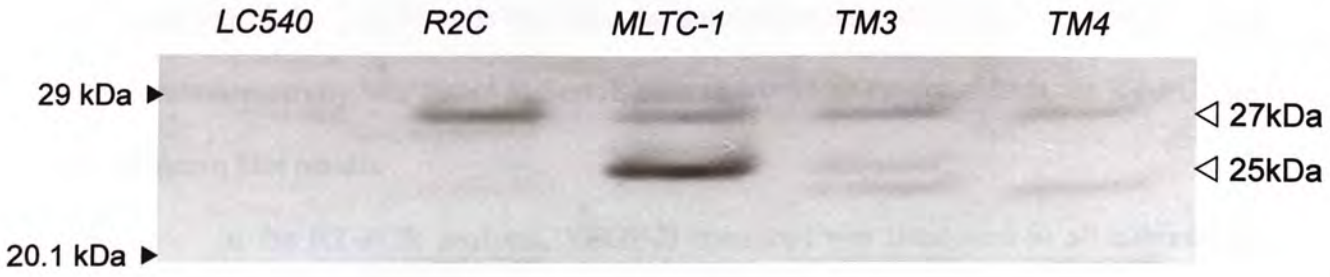


Fig.3.10 Western blot analysis of VEGF-C protein expression in various testicular cell lines. Equal amount of total protein from cell lysate of each testicular cell lines was size fractionated on a 15% SDS-polyacrylamide gel under reducing conditions, transferred to a PVDF membrane, and immunoblotted with a rabbit polyclonal anti-VEGF-C antibody. The blot was then incubated with horseradish peroxidase-conjugated anti-rabbit IgG and visualized by the ECL detection system. The antibody recognized proteins with apparent molecular mass of 27 kDa and 25 kDa. The positions of molecular mass markers were shown on the left.

3.1.4 VEGF-D

In immunohistochemical staining, VEGF-D immunoreactivity was mainly localized in the Leydig cells and vascular smooth muscle cells (Fig.3.1G and H). Little immunoreactivity was found in Sertoli cells as would be expected from the RT-PCR and Western blot results.

In the RT-PCR analysis, VEGF-D transcript was illustrated in all cultured cell types (except in germ cell) and rat testis, and they showed different expression patterns and intensities (Fig.3.11). The size of the PCR product corresponded to what would be expected (i.e. 233 bp) from the amplification of VEGF-D transcript. RT-PCR results illustrated that VEGF-D transcript was expressed in Leydig cell and Sertoli cell.

In Western blot analysis, the polyclonal antibody recognized proteins with apparent molecular mass of 46 kDa, 37 kDa, 30 kDa, 23 kDa and 16 kDa representing different processed forms of VEGF-D in cultured primary testicular cells and testis. In testicular cell lines, proteins with apparent molecular mass of 30 kDa, 28 kDa, 23 kDa and 16 kDa representing different processed forms of VEGF-D were found. They all showed different abundance and expression patterns (Fig.3.12 and 3.13). According to the Western blot results which were based on the use of the same amount of total protein, Leydig cell appeared to be the major source of VEGF-D protein (Fig.3.12).

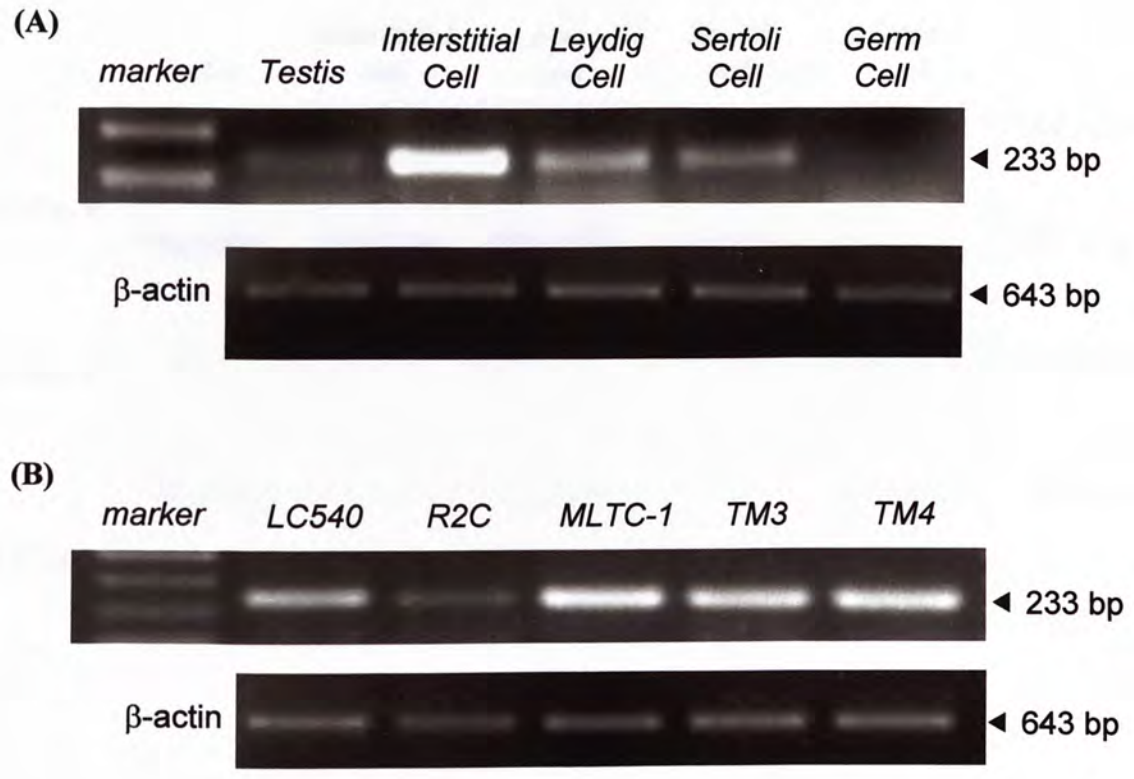


Fig.3.11 RT-PCR analysis of VEGF-D mRNA expression in rat testis, testicular primary cells (A) and testicular cell lines (B). A PCR product corresponding in size to what would be expected (i.e. 233 bp) form the amplification of VEGF-D transcript was found. The amount of template used in PCR was adjusted based on prior amplification of an internal control, β-actin, with a product size of 643bp (lower panel) which gave similar signal intensities for each samples.

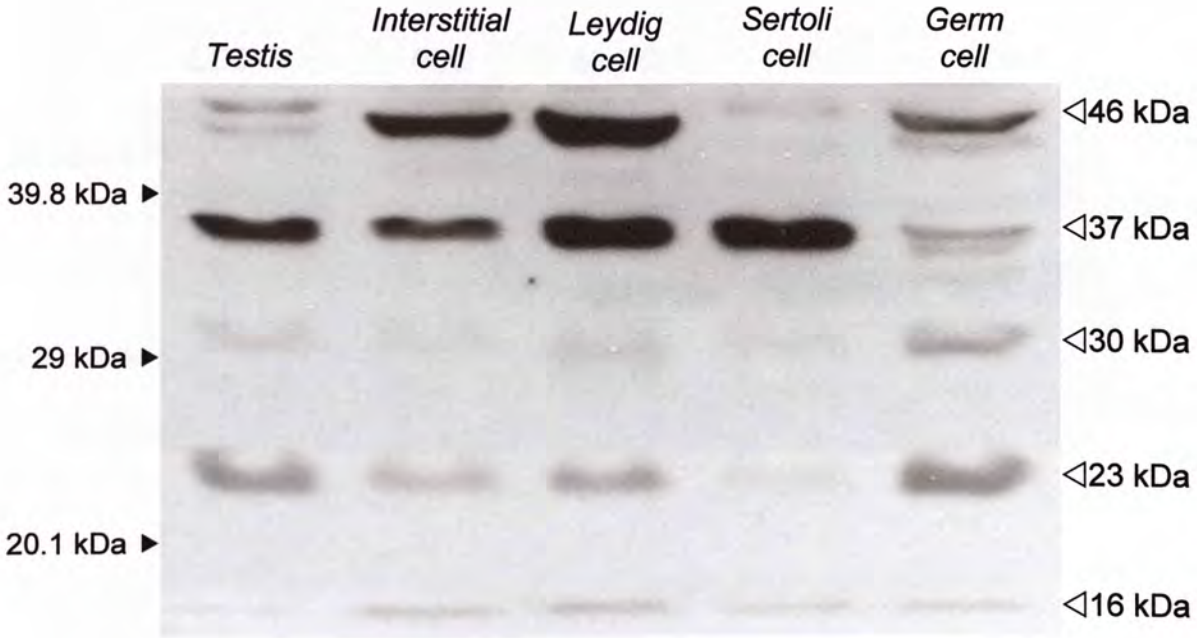


Fig.3.12 Western blot analysis of VEGF-D protein expression in rat testis and primary testicular cells. Equal amount of total protein from each tissue or cell lysate was size fractionated on a 15% SDS-polyacrylamide gel under reducing conditions, transferred to a PVDF membrane, and immunoblotted with a rabbit polyclonal anti-VEGF-D antibody. The blot was then incubated with horseradish peroxidase-conjugated anti-rabbit IgG and visualized by the ECL detection system. The antibody recognized proteins with apparent molecular mass of 46 kDa, 37 kDa, 30 kDa, 23 kDa and 16 kDa representing different processed forms of VEGF-D. The positions of molecular mass markers were shown on the left.

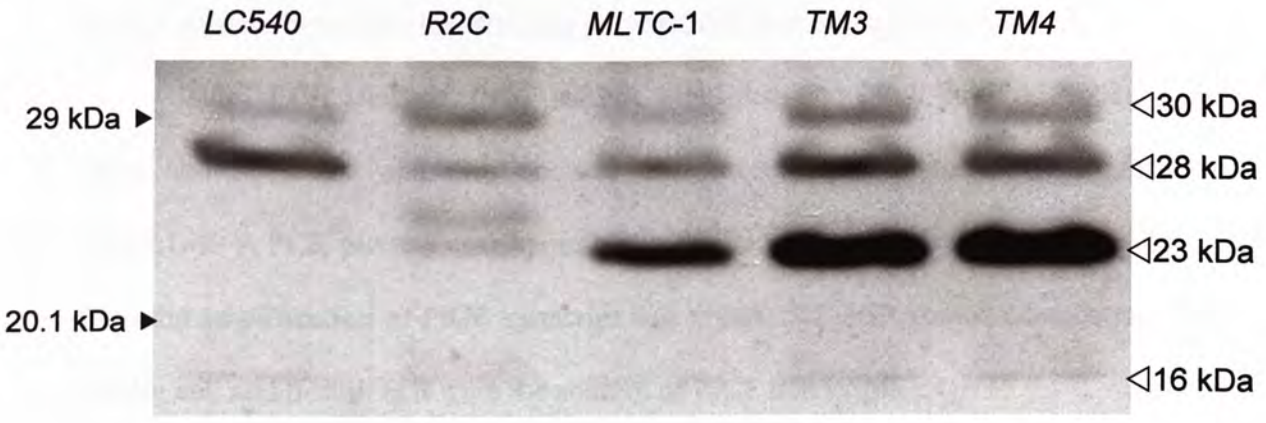


Fig.3.13 Western blot analysis of VEGF-D protein expression in various testicular cell lines. Equal amount of total protein from cell lysate of each testicular cell line was size fractionated on a 15% SDS-polyacrylamide gel under reducing conditions, transferred to a PVDF membrane, and immunoblotted with a rabbit polyclonal anti-VEGF-D antibody. The blot was then incubated with horseradish peroxidase-conjugated anti-rabbit IgG and visualized by the ECL detection system. The antibody recognized proteins with apparent molecular mass of 30 kDa, 28 kDa, 23 kDa and 16 kDa representing different processed forms of VEGF-D. The positions of molecular mass markers were shown on the left.

3.1.5 PlGF

Owing to no suitable commercially available PlGF antibody that could be used for the immunohistochemical staining purpose, this part of study was omitted.

In RT-PCR analysis, PlGF mRNA was illustrated in all cultured testicular cell types and rat testis, and they showed different expression patterns and intensities (Fig.3.14). A PCR product corresponding in size to what would be expected (i.e. 383 bp) from the amplification of PlGF transcript was found. RT-PCR results demonstrated that Leydig cell and Sertoli cell were the sources of PlGF transcripts.

Western blot analysis showed one major protein band with apparent molecular mass of 27 kDa in all cultured cell types and testis with different abundance and expression patterns (Fig.3.15 and 3.16). Under reducing conditions, this protein band might correspond to the monomer of PlGF. Moreover, another protein band with an apparent molecular mass of 45 kDa was also found (Fig.3.15).

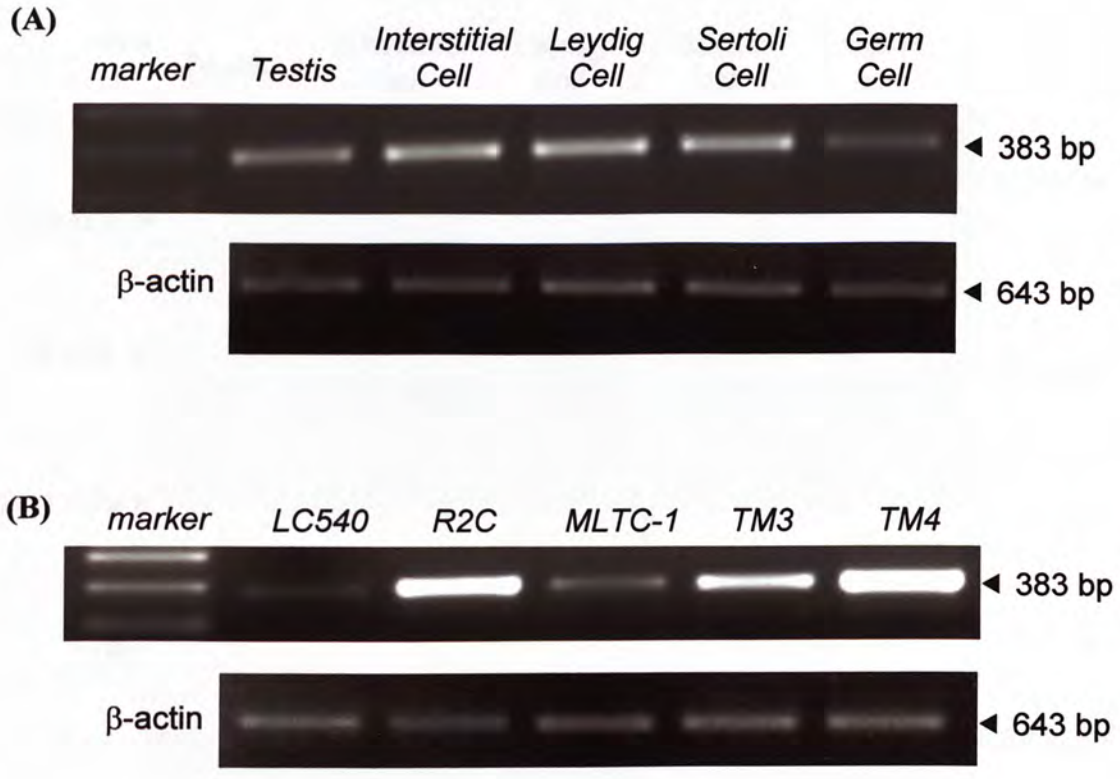


Fig.3.14 RT-PCR analysis of PlGF mRNA expression in rat testis, testicular primary cells (A) and testicular cell lines (B). A PCR product corresponding in size to what would be expected (i.e. 383 bp) from the amplification of PlGF transcript, was found. The amount of template used in PCR was adjusted based on prior amplification of an internal control, β-actin, with a product size of 643bp (lower panel) which gave similar signal intensities for each samples.

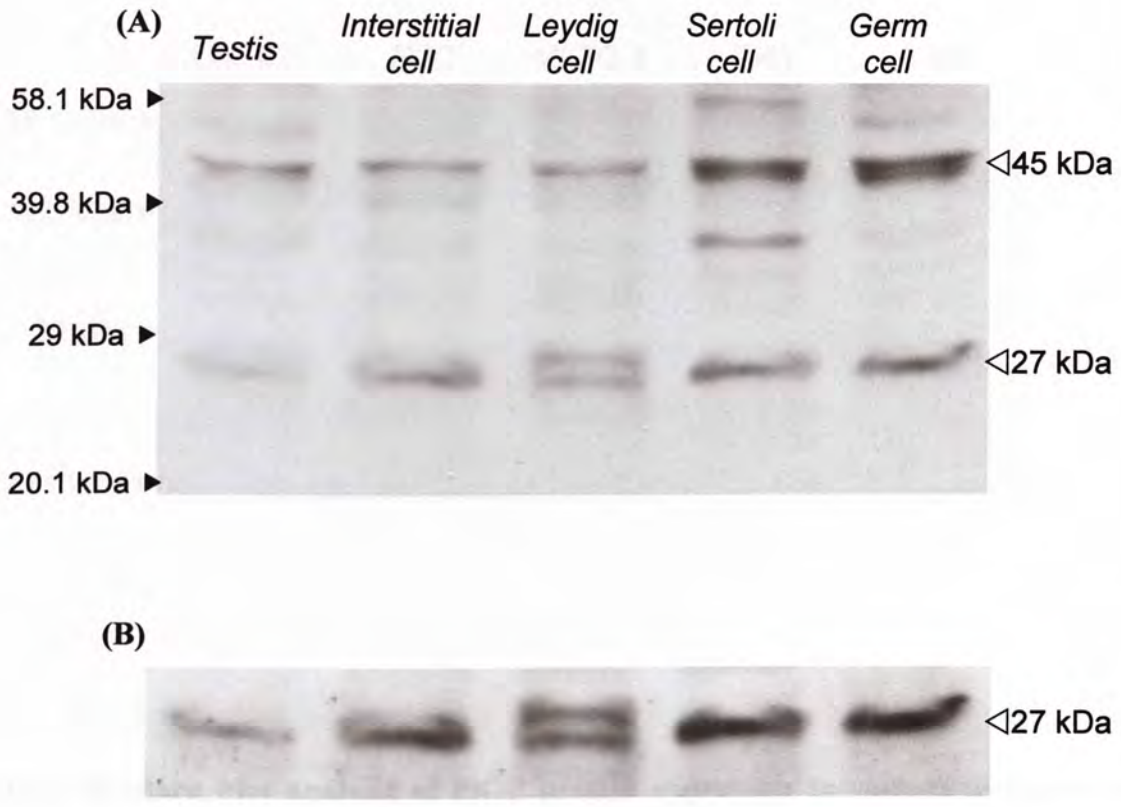


Fig.3.15 Western blot analysis of PlGF protein expression in rat testis and primary testicular cells. **(A)** Equal amount of total protein from tissue or cell lysate was size fractionated on a 12.5% SDS-polyacrylamide gel under reducing conditions, transferred to a PVDF membrane, and immunoblotted with a goat polyclonal anti-PlGF antibody. The blot was then incubated with horseradish peroxidase-conjugated anti-goat IgG and visualized by the ECL detection system. The antibody recognized proteins with an apparent molecular mass of 27 kDa, and 45 kDa. An extra band (28 kDa) detected in Leydig cell lysate might represent a glycosylated form of PlGF. The positions of molecular mass markers were shown on the left. **(B.)** A clearer image of the bands of 27 kDa was showed by the same blot with longer exposure time.

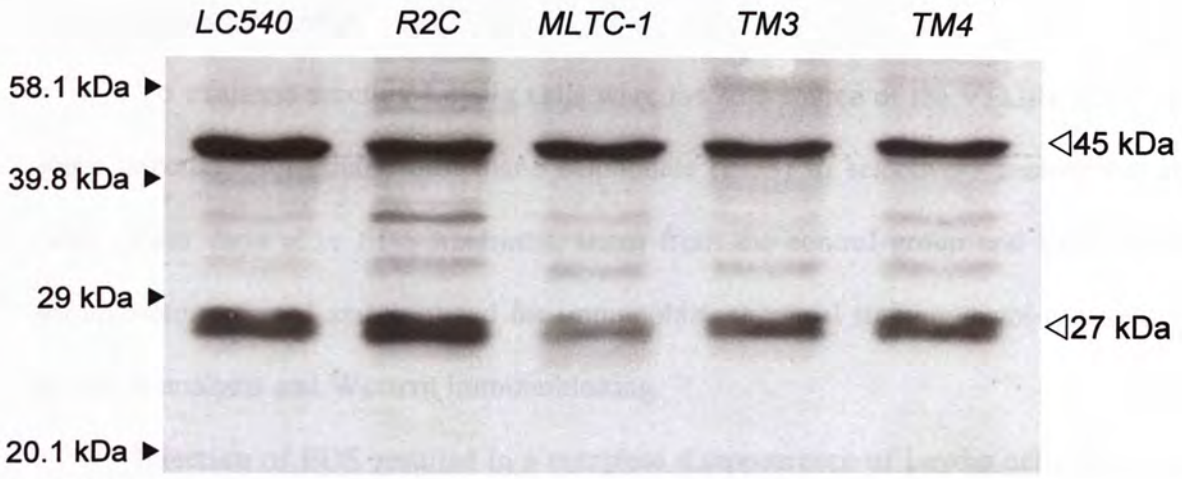


Fig.3.16 Western blot analysis of PlGF protein expression in various testicular cell lines. Equal amount of total protein from cell lysate of each cell line was size fractionated on a 12.5% SDS-polyacrylamide gel under reducing conditions, transferred to a PVDF membrane, and immunoblotted with a goat polyclonal anti-PlGF antibody. The blot was then incubated with horseradish peroxidase-conjugated anti-goat IgG and visualized by the ECL detection system. The antibody recognized proteins with an apparent molecular mass of 27 kDa and 45 kDa. The positions of molecular mass markers were shown on the left.

3.2 Effect of Leydig cell depletion on VEGFs expression in the rat testis

3.2.1 Effect on VEGF-A

To examine whether Leydig cells were the sole source of the VEGFs, adult rats were injected with ethane dimethane sulphonate (EDS) to selectively destroy Leydig cells. Four days after EDS treatment, testes from the control group and EDS-treated group were removed and prepared for immunohistochemical staining, semi-quantitative RT-PCR analysis and Western immunoblotting.

Injection of EDS resulted in a complete disappearance of Leydig cells at 4 days after treatment (Fig.3.17B, D, F and H). This was indicated by fewer numbers of cells and enlarged spaces in the testicular interstitium.

From the immunostaining for VEGF-A, there was a near complete loss of VEGF-A immunoreactivity in the testicular interstitium, whereas the immunoreactivity in Sertoli cells remained strong and intense (Fig.3.17A and B). Besides, vascular smooth muscle cells were weakly stained indicating that the expression of VEGF-A in both Sertoli cells and vascular smooth muscle cells were not affected by the EDS treatment.

RT-PCR analysis demonstrated the changes in four spliced variants of VEGF-A mRNA: 645bp, 573bp, 513bp and 441bp, representing VEGF-A_{188}, VEGF-A_{164}, VEGF-A_{144} and VEGF-A_{120} respectively (Fig.3.18A). Combined data of densitometric quantification taken from a number of animals indicated that the levels of all four spliced variants dropped with VEGF-A_{144} reaching a statistical significance (Fig.3.18B).

Western immunoblotting demonstrated the changes in three isoforms of VEGF-A: VEGF-A_{188}, VEGF-A_{164} and VEGF-A_{120} (Fig.3.19A). The combined data of densitometric quantification taken from a number of animals indicated that VEGF-A_{164} and VEGF-A_{120} levels decreased after treatment, and VEGF-A_{164} showed a complete

disappearance from the blot (Fig.3.19B). On the contrary, expression of VEGF-A_{188} remained unaffected by the EDS treatment. These results illustrated that Leydig cells would represent the major source of VEGF-A_{164} and VEGF-A_{120} isoforms in the testis.

3.2.2 Effect on VEGF-B

Immunohistochemical study showed a complete loss of VEGF-B immunoreactivity from the testicular interstitium after EDS treatment, whereas the immunoreactivity in Sertoli cells became more apparent after treatment (Fig.3.17C and D).

RT-PCR analysis demonstrated the changes in the levels of the two spliced variants of VEGF-B mRNA: 425bp and 324bp, representing VEGF-B_{186} and VEGF-B_{167} respectively (Fig.3.20A). The combined data of densitometric quantification taken from a number of animals showed that the levels of both spliced variants dropped with statistical significance (Fig.3.20B).

Western immunoblotting analysis demonstrated the changes in two isoforms of VEGF-B, VEGF-B_{186} and VEGF-B_{167} (Fig.3.21A). The combined data of densitometric quantification taken from a number of animals indicated that VEGF-B_{186} and VEGF-B_{167} peptide levels decreased after treatment but only the change in VEGF-B_{167} was statistically significant (Fig.3.21B). These results demonstrated that Leydig cell was probably a major source of VEGF-B_{167} and VEGF-B_{186}. Comparing to the immunostaining study, Sertoli cells could be an alternative source of VEGF-B_{186} and VEGF-B_{167}, especially following the destruction of Leydig cells and / or the withdrawal of androgen after Leydig cells depletion.

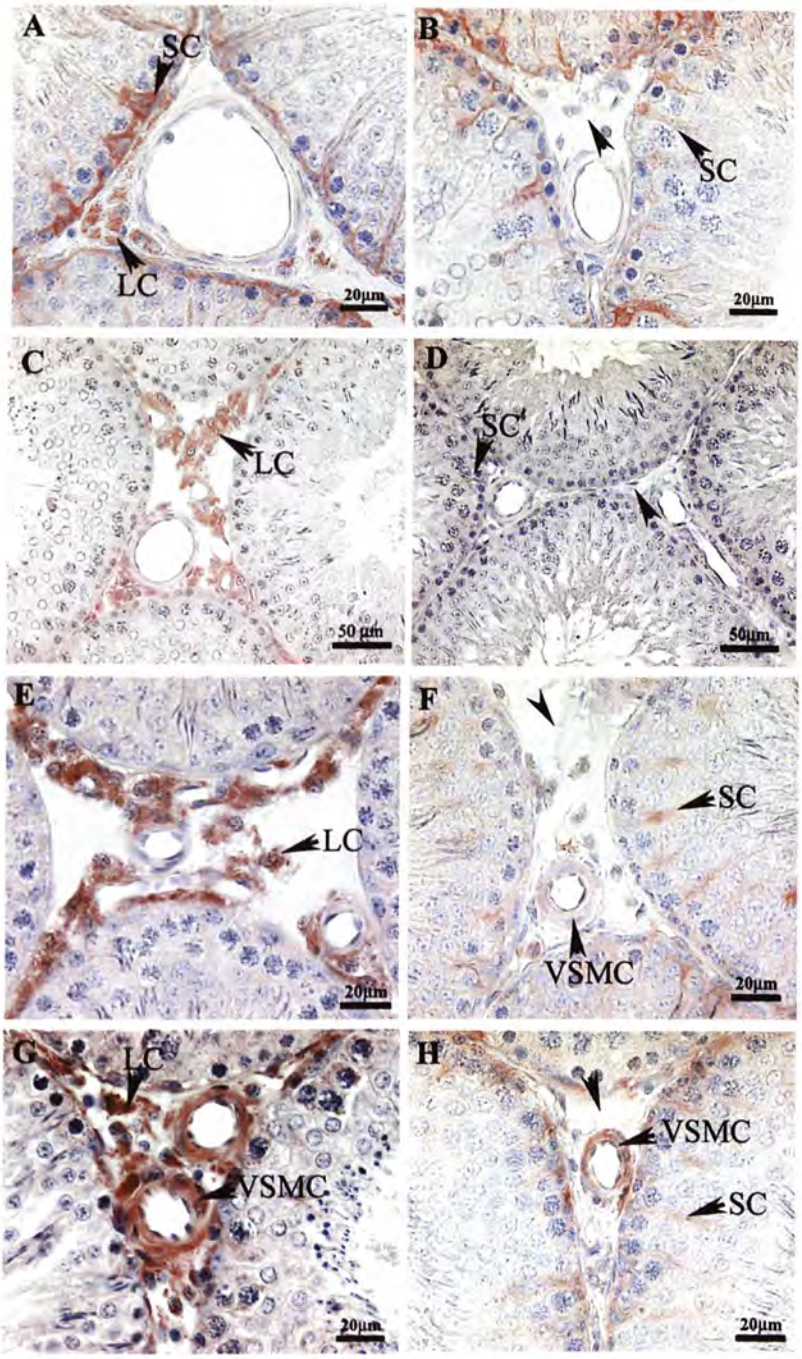


Fig.3.17 Immunohistochemical staining of VEGFs in the normal adult rat testes (A, C, E and G) and testes of rats at 4 days after ethane dimethane sulphonate (EDS) treatment (B, D, F and H). Positive VEGFs immunoreactivity was indicated by the red colour and arrows in tissue sections that were counterstained with haematoxylin. Note that in testes treated with EDS, there were very few interstitial cells present in the interstitium. (**A-B**) showed the immunoreactivity of VEGF-A before and after treatment. (**C-D**) showed the immunoreactivity of VEGF-B before and after treatment. (**E-F**) showed the immunoreactivity of VEGF-C before and after treatment. (**G-H**) showed the immunoreactivity of VEGF-D before and after treatment. *Abbreviations stand for: LC: Leydig cells, SC: Sertoli cells and VSMC: vascular smooth muscle cells.*

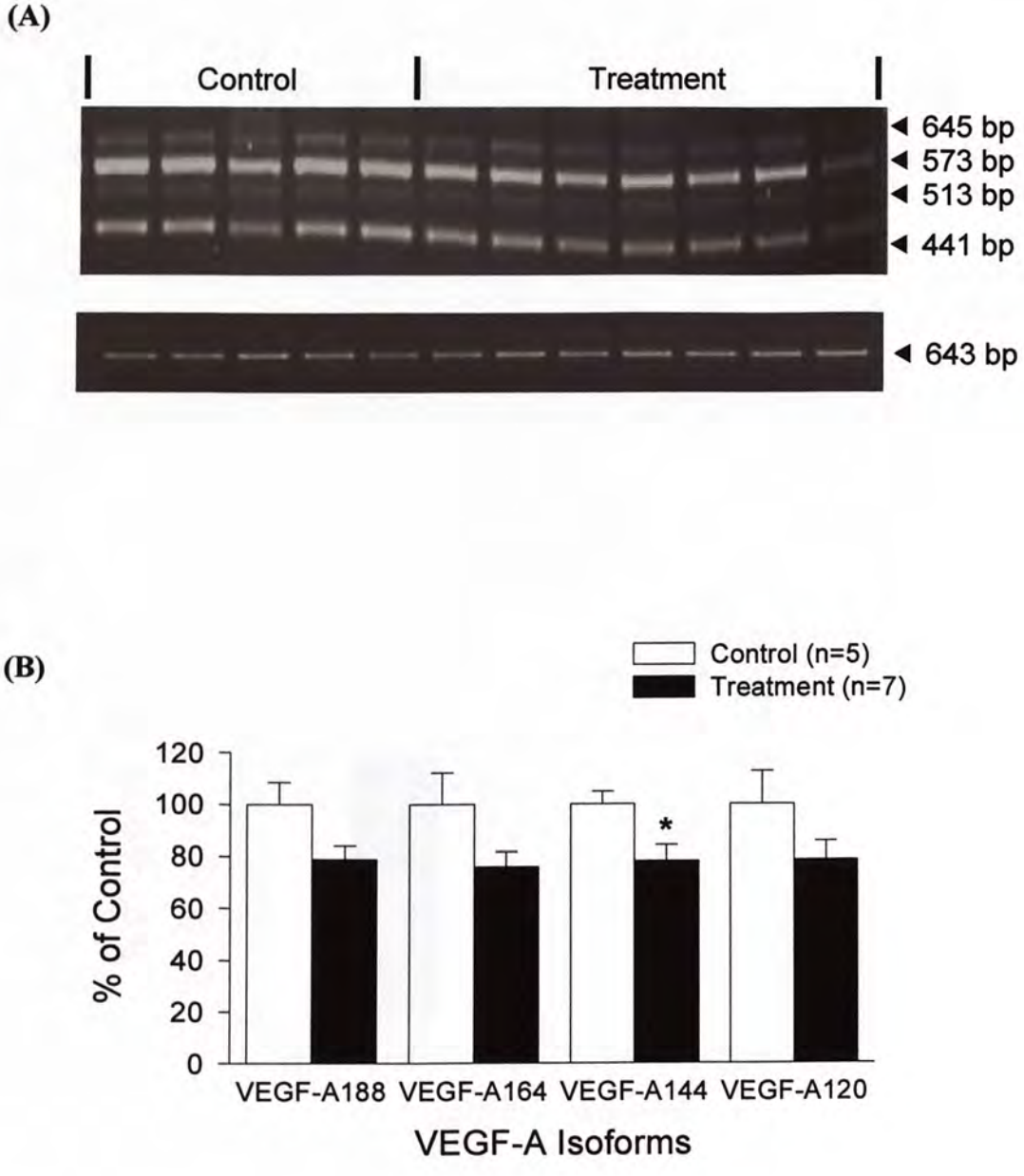


Fig.3.18 Effect of Leydig cell depletion by ethane dimethane sulphonate (EDS) on VEGF-A mRNA expression level in the adult rat testis. **(A)** Representative semi-quantitative RT-PCR result showing the effect of treatment on the levels of the four major spliced variants of VEGF-A mRNA (645bp, 573bp, 513bp and 441bp) representing VEGF-A_{188}, VEGF-A_{164}, VEGF-A_{144} and VEGF-A_{120} respectively. The amplification of β-actin was included as an internal control to adjust the amount of template used. **(B)** Combined data from the densitometric quantification of the signals of four VEGF-A variants, which were normalized against the control as 100%. Data represent mean ± SE of 5 animals per control group and 7 animals per treatment group. *$P<0.05$, by Mann-Whitney rank sum test for comparison with the corresponding control.

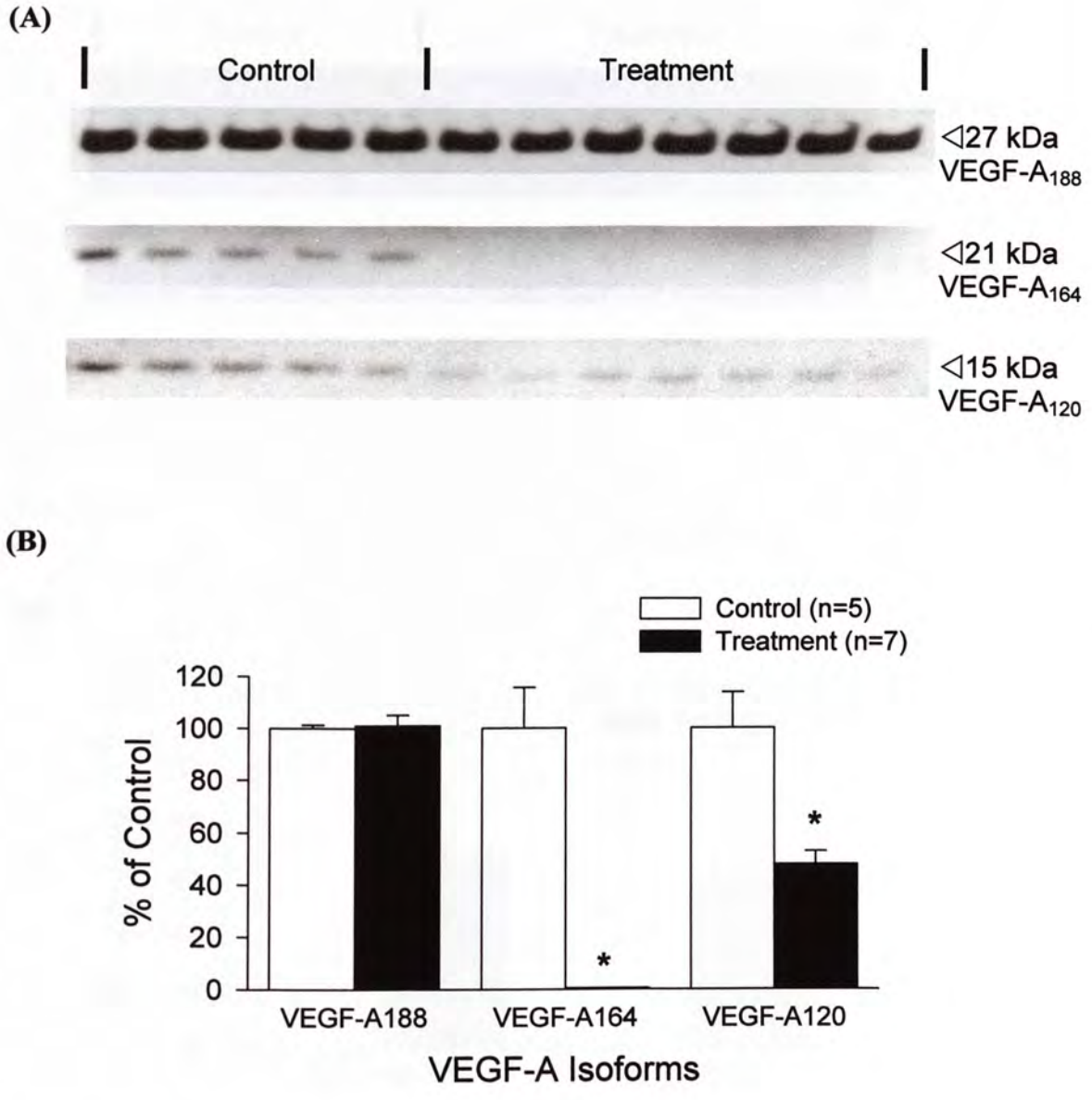


Fig.3.19 Effect of Leydig cell depletion by ethane dimethane sulphonate (EDS) on VEGF-A protein expression level in the adult rat testis. **(A)** Representative Western blots showed the effect of treatment on the levels of the three VEGF-A isoforms in the testes. **(B)** Combined data from the densitometric quantification of the signals of three VEGF-A isoforms, which were normalized against the control as 100%. Data represent mean ± SE of 5 animals per control group and 7 animals per treatment group. *P<0.05, by Mann-Whitney rank sum test for comparison with the corresponding control.

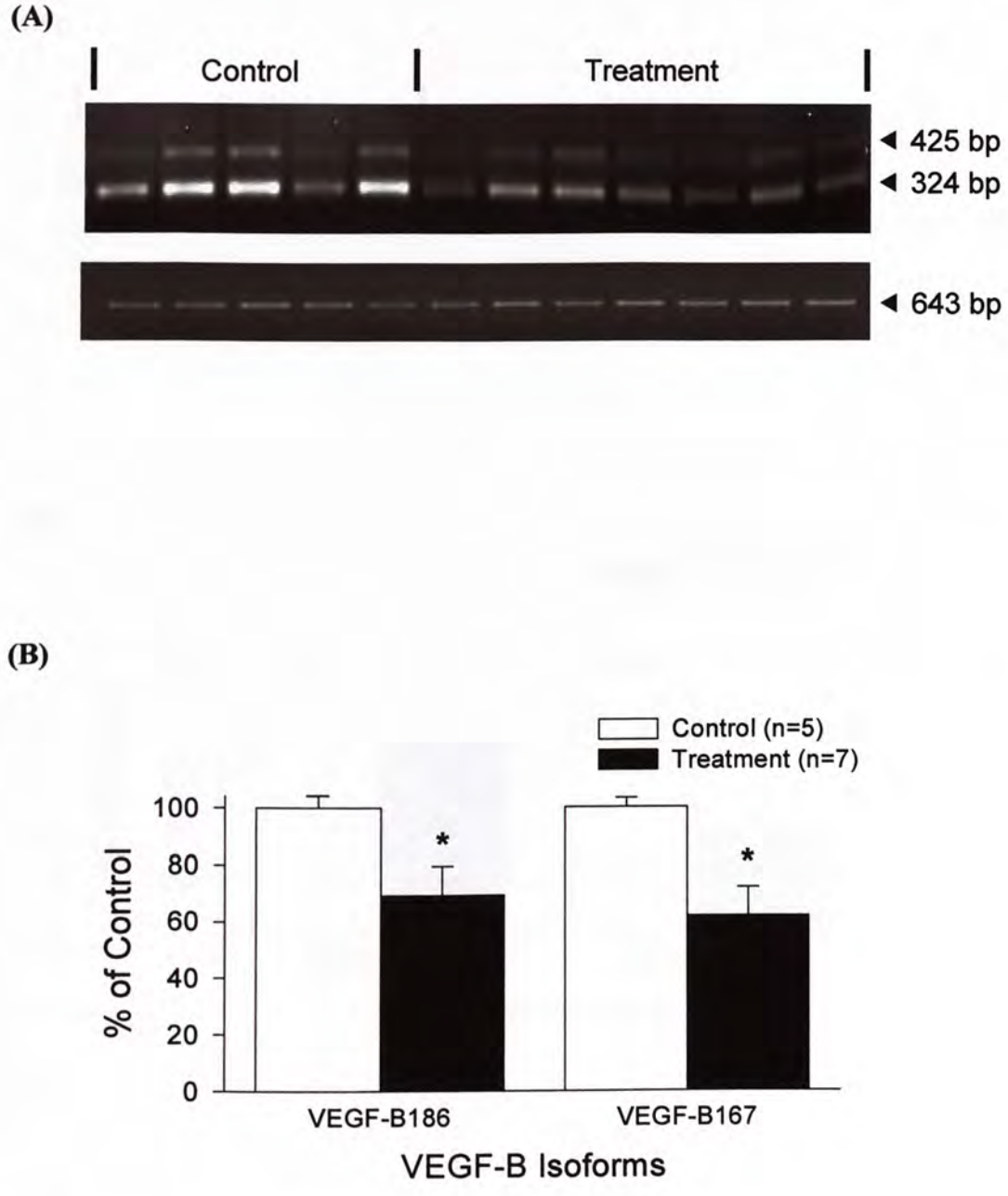


Fig.3.20 Effect of Leydig cell depletion by ethane dimethane sulphonate (EDS) on VEGF-B mRNA expression level in the adult rat testis. **(A)** Representative semi-quantitative RT-PCR result showing the effect of treatment on the levels of the two spliced variants of VEGF-B mRNA (425 bp and 324 bp) representing VEGF-B_{186} and VEGF-B_{167} respectively. The amplification of β-actin was included as an internal control to adjust the amount of template used. **(B)** Combined data from the densitometric quantification of the signals of two VEGF-B variants, which were normalized against the control as 100%. Data represent mean ± SE of 5 animals per control group and 7 animals per treatment group. *$P<0.05$, by Mann-Whitney rank sum test for comparison with the corresponding control.

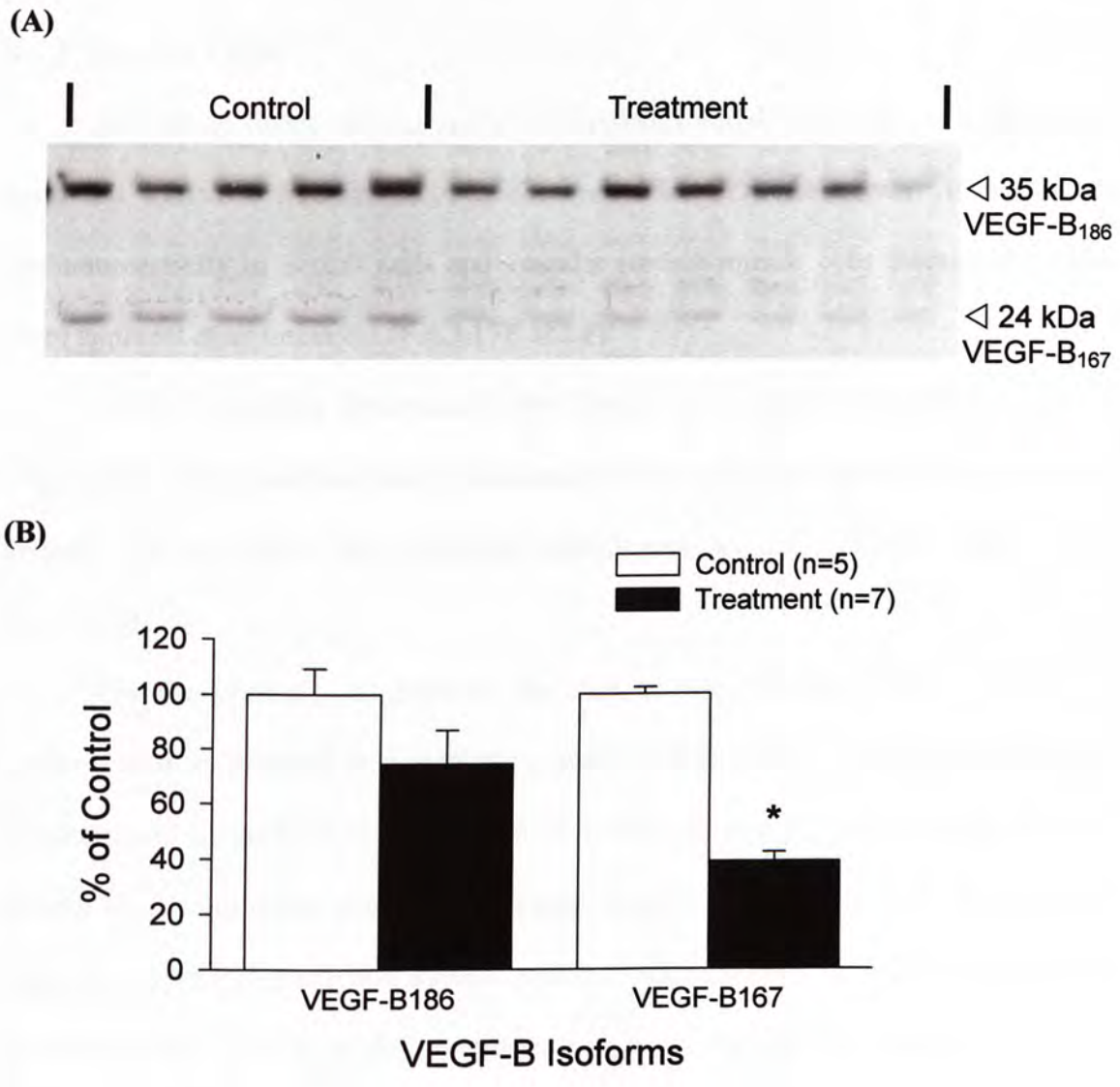


Fig.3.21 Effect of Leydig cell depletion by ethane dimethane sulphonate (EDS) on VEGF-B protein expression level in the adult rat testis. (A) A representative Western blot showed the effect of treatment on the level of the two VEGF-B isoforms in the testes. **(B)** Combined data from the densitometric quantification of the signals of two VEGF-B isoforms, which were normalized against the control as 100%. Data represent mean ± SE of 5 animals per control group and 7 animals per treatment group. *P<0.05, by Mann-Whitney rank sum test for comparison with the corresponding control.

3.2.3 Effect on VEGF-C

In immunohistochemical study, a complete loss of VEGF-C immunoreactivity from the testicular interstitium was observed after EDS treatment. However, the immunoreactivity in Sertoli cells and vascular smooth muscle cells seemed to become more apparent after treatment (Fig.3.17E and F).

RT-PCR analysis demonstrated the changes in the level of the VEGF-C mRNA (Fig.3.22A). The combined data of densitometric quantification taken from a number of animals did not show any statistical significance in the VEGF-C mRNA level (Fig.3.22B).

On the contrary, a dramatic decrease in the 27 kDa VEGF-C level was demonstrated in Western immunoblotting analysis (Fig.3.23A). The combined data of densitometric quantification taken from a number of animals showed that VEGF-C protein level went down dramatically to near disappearance (Fig.3.23B). These results suggested that Leydig cell was a major source of VEGF-C peptide, and in particular the 27 kDa protein. Results of the immunostaining study showed that despite the dramatic fall in the level of the VEGF-C 27 kDa peptide, some VEGF-C immunoreactivities remained in Sertoli cells and vascular smooth muscle cells.

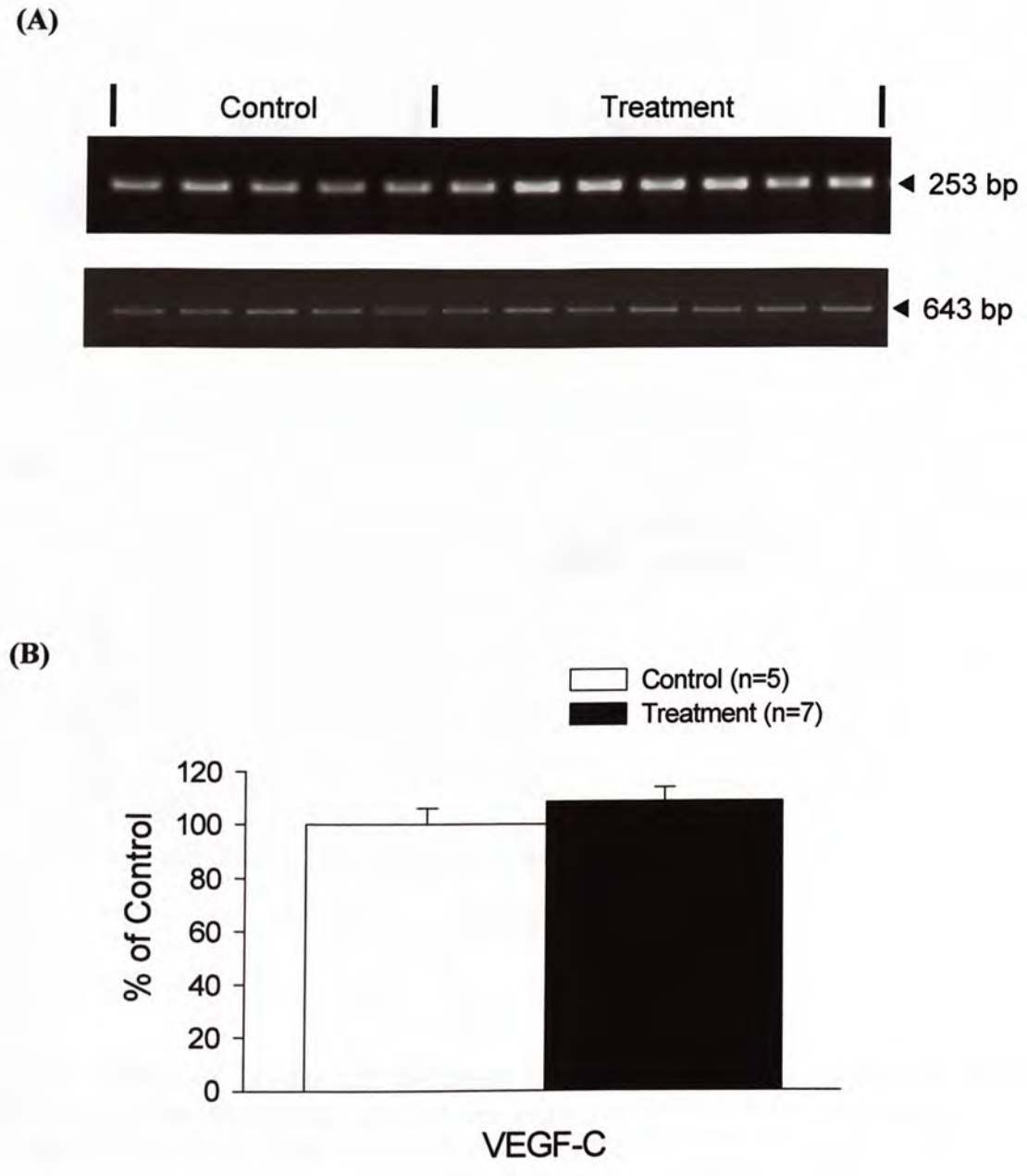


Fig.3.22 Effect of Leydig cell depletion by ethane dimethane sulphonate (EDS) on VEGF-C mRNA expression level in the adult rat testis. **(A)** Representative semi-quantitative RT-PCR result showing the effect of treatment on the level of the VEGF-C mRNA (253 bp). The amplification of β-actin was included as an internal control to adjust the amount of template used. **(B)** Combined data from the densitometric quantification of the signals of VEGF-C mRNA, which were normalized against the control as 100%. Data represent mean ± SE of 5 animals per control group and 7 animals per treatment group. *P<0.05, by Mann-Whitney rank sum test for comparison with the corresponding control.

(A)

(B)

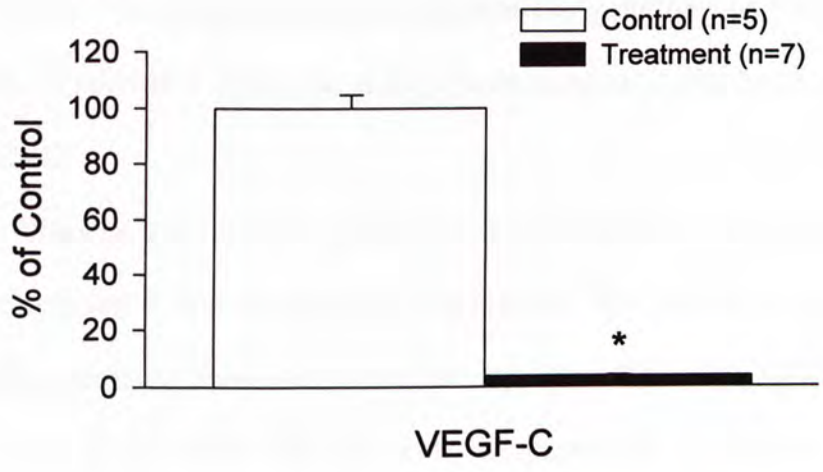


Fig.3.23 Effect of Leydig cell depletion by ethane dimethane sulphonate (EDS) on VEGF-C protein expression level in the adult rat testis. (A) A representative Western blot showed the effect of treatment on the level of the VEGF-C in the testes. **(B)** Combined data from the densitometric quantification of the signals of VEGF-C, which were normalized against the control as 100%. Data represent mean ± SE of 5 animals per control group and 7 animals per treatment group. *$P<0.05$, by Mann-Whitney rank sum test for comparison with the corresponding control.

3.2.4 Effect on VEGF-D

Immunohistochemical study showed a complete disappearance of VEGF-D immunoreactivity from the testicular interstitium following the selective destruction of Leydig cell after EDS treatment, whereas the immunoreactivity in vascular smooth muscle cells remained. Besides, immunoreactivity in Sertoli cells seemed to become more apparent after treatment (Fig.3.17G and H).

RT-PCR analysis demonstrated the changes in the level of the VEGF-D mRNA (Fig.3.24A). The combined data of densitometric quantification taken from a number of animals showed that there was a significant increase in the level of VEGF-D mRNA (Fig.3.24B).

Western immunoblotting analysis demonstrated the changes in the levels of the three processed forms of VEGF-D (Fig.3.25A). The combined data of densitometric quantification taken from a number of animals showed that the signal intensity for the 37 kDa form of VEGF-D fell but with no statistically significant level (Fig.3.25B). However, EDS treatment did not produce any change in 23 kDa protein level. In contrast, the 16 kDa peptide level increased to a significant level indicating that removal of Leydig cells hence the source of androgen caused stimulation to certain cell type in the testis to produce more 16 kDa peptide of VEGF-D. These results implied that Leydig cell might be a source of 37 kDa processed forms of VEGF-D. On the contrary, the expression of 16 kDa form of VEGF-D could come from other cellular sources and be upregulated following the removal of androgen from destroying the Leydig cells. Based on the results of immunostaining study, Sertoli cells and vascular smooth muscle cells appeared to be an alternative source of VEGF-D peptides.

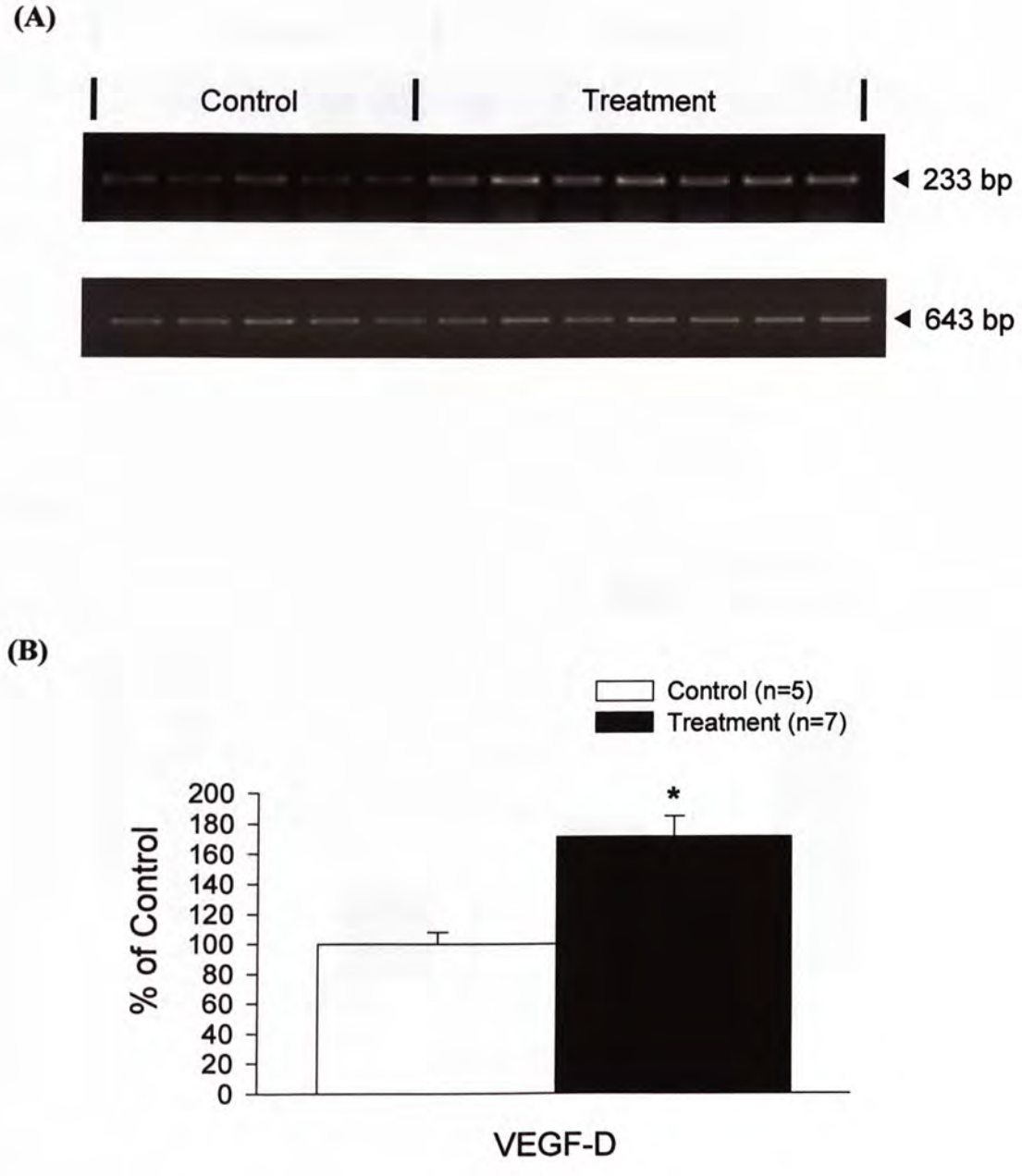


Fig.3.24 Effect of Leydig cell depletion by ethane dimethane sulphonate (EDS) on VEGF-D mRNA expression level in the adult rat testis. (A) Representative semi-quantitative RT-PCR result showing the effect of treatment on the level of the VEGF-D mRNA (233 bp). The amplification of β-actin was included as an internal control to adjust the amount of template used. **(B)** Combined data from the densitometric quantification of the signals of VEGF-D mRNA, which were normalized against the control as 100%. Data represent mean ± SE of 5 animals per control group and 7 animals per treatment group. *$P<0.05$, by Mann-Whitney rank sum test for comparison with the corresponding control.

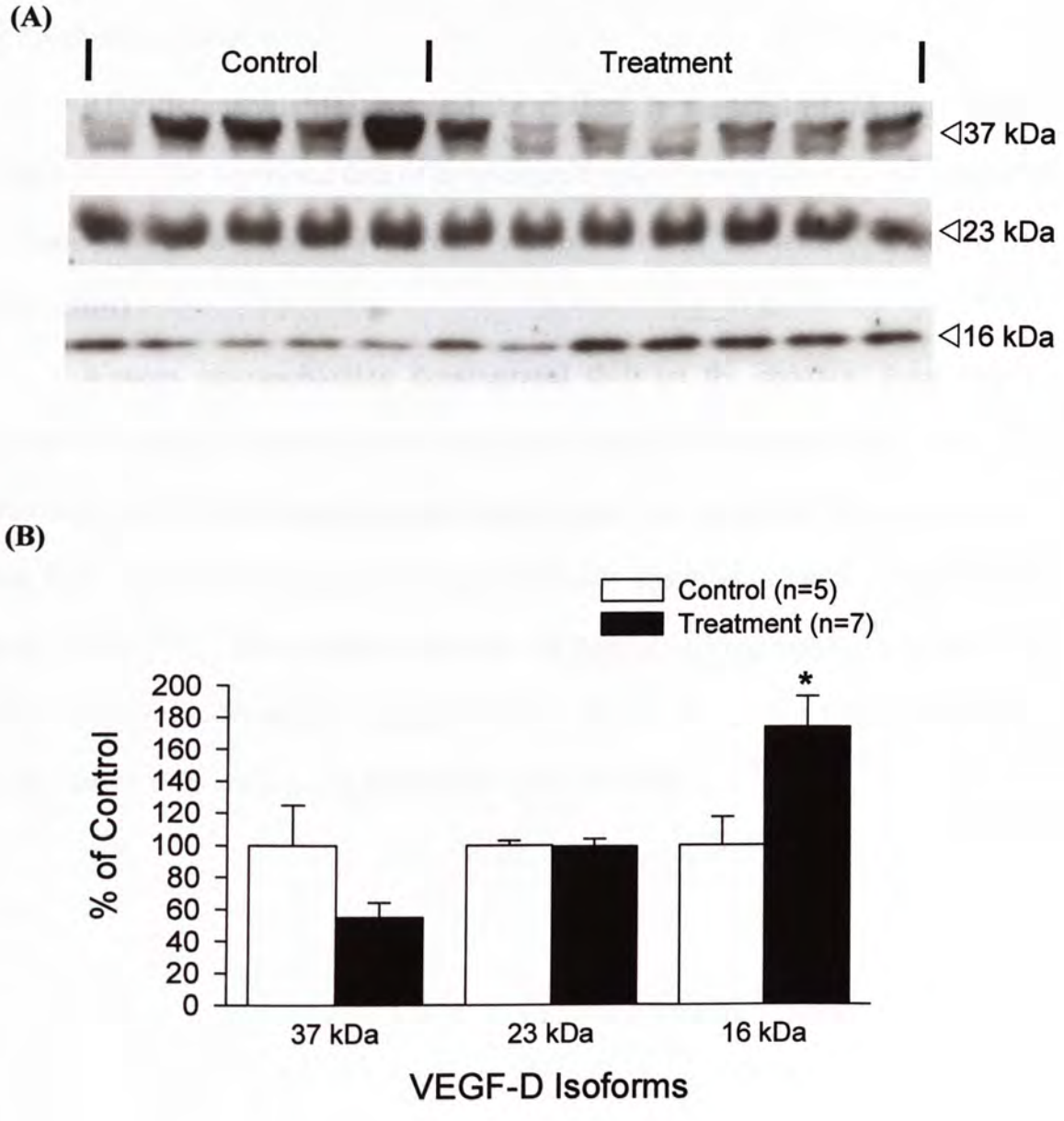


Fig.3.25 Effect of Leydig cell depletion by ethane dimethane sulphonate (EDS) on VEGF-D protein expression level in the adult rat testis. **(A)** Representative Western blots showed the effect of treatment on the levels of the three VEGF-D isoforms in the testes. **(B)** Combined data from the densitometric quantification of the signals of three VEGF-D isoforms, which were normalized against the control as 100%. Data represent mean ± SE of 5 animals per control group and 7 animals per treatment group. *P<0.05, by Mann-Whitney rank sum test for comparison with the corresponding control.

3.2.5 Effect on PlGF

RT-PCR analysis demonstrated the changes in the level of the PlGF mRNA (Fig.3.26A). The combined data of densitometric quantification taken from a number of animals showed a significant increase was observed in the level of PlGF mRNA (Fig.3.26B).

Western immunoblotting demonstrated that, on the contrary, there was no significant change in PlGF protein level before and after treatment (Fig.3.27A). The combined data of densitometric quantification taken from a number of animals showed that EDS treatment did not produce any statistically significant change in PlGF protein level (Fig.3.27B). These results indicated that Leydig cell was not the only source of PlGF in the testis. From previous RT-PCR and Western blot results (Fig.3.14, 3.15 and 3.16), Sertoli cells could be an alternative source of PlGF.

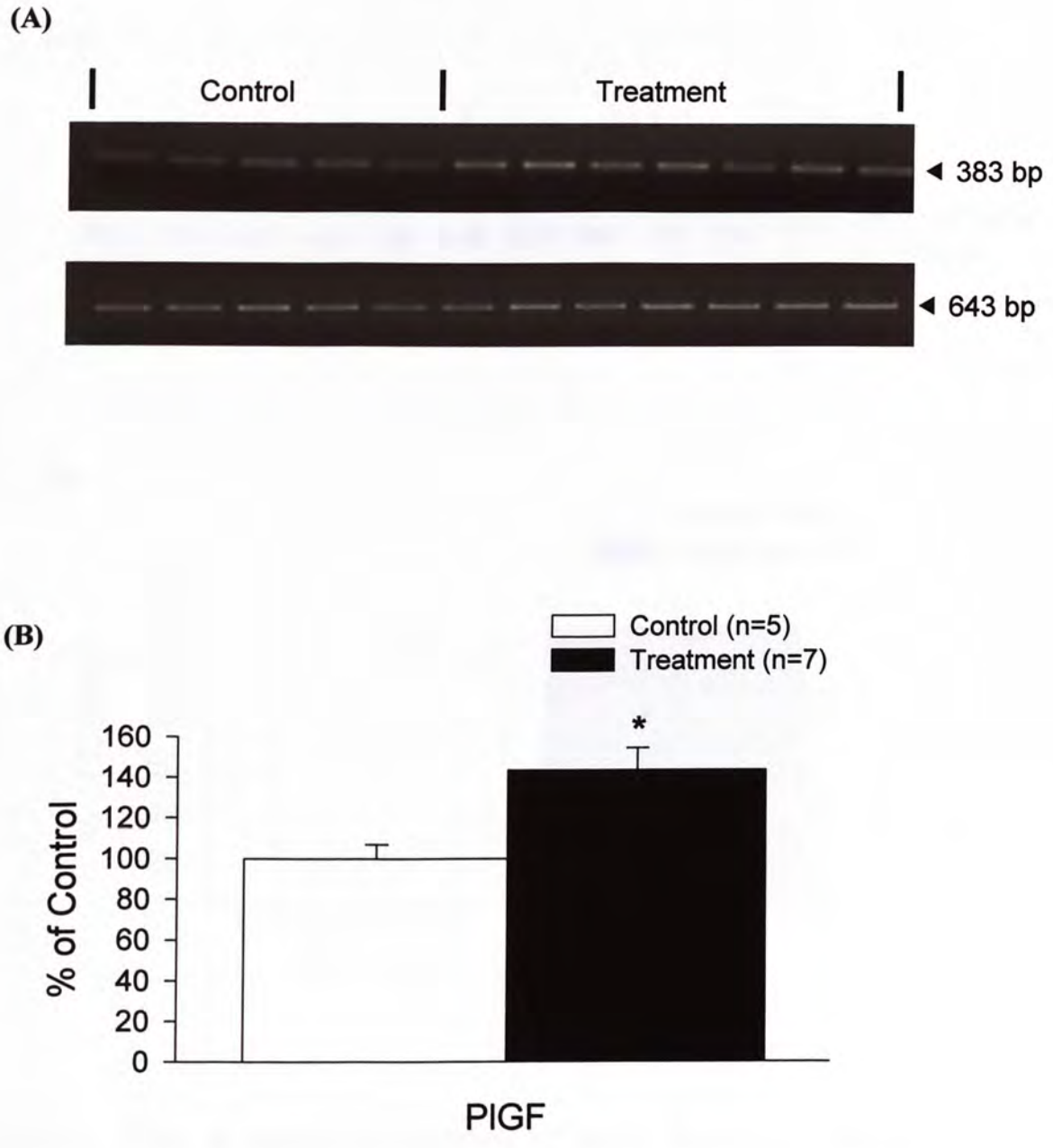


Fig.3.26 Effect of Leydig cell depletion by ethane dimethane sulphonate (EDS) on PlGF mRNA expression level in the adult rat testis. (A) Representative semi-quantitative RT-PCR result showing the effect of treatment on the level of the PlGF mRNA (383 bp). The amplification of β-actin was included as an internal control to adjust the amount of template used. **(B)** Combined data from the densitometric quantification of the signals of PlGF mRNA, which were normalized against the control as 100%. Data represent mean ± SE of 5 animals per control group and 7 animals per treatment group. *P<0.05, by Mann-Whitney rank sum test for comparison with the corresponding control.

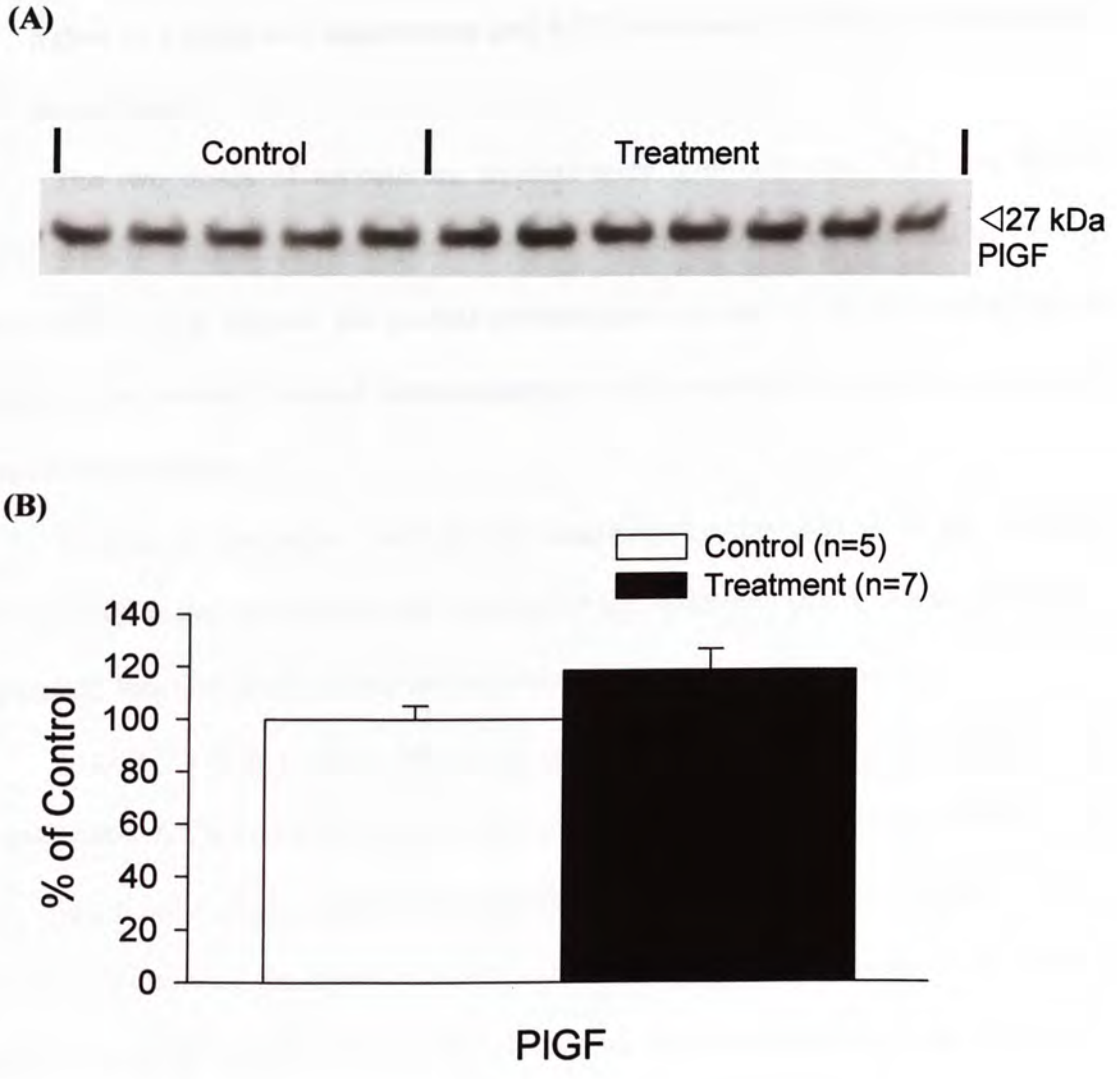


Fig.3.27 Effect of Leydig cell depletion by ethane dimethane sulphonate (EDS) on PlGF protein expression level in the adult rat testis. **(A)** A representative Western blot showed the effect of treatment on the level of the PlGF in the testes. **(B)** Combined data from the densitometric quantification of the signals of PlGF, which were normalized against the control as 100%. Data represent mean ± SE of 5 animals per control group and 7 animals per treatment group.

3.3 Effect of Leydig cell suppression and hCG stimulation on VEGFs expression in the rat testis

The two doses of testosterone implant were used such that the 3 cm implant would achieve normal physiological levels of serum testosterone but fail to provide adequate hormonal support for normal spermatogenesis, and the 25 cm implant would maintain near normal levels of spermatogenesis with a supraphysiological concentration of serum testosterone.

In view of the earlier findings demonstrating Leydig cells to be the source of several VEGFs, the following study was carried out to determine how the suppression of Leydig cell function would affect the expression of VEGFs in the testis.

In both 3 cm and 25 cm implant groups, the testis weight decreased significantly to reach about 65 % and 90 % of the value found in the control group respectively (Table 3.1). Such a fall in testis weight mainly represented the loss of spermatogenic activity in the testes. Following the injection of hCG, there was an increasing tendency in the testis weight among the normal rats and the testosterone-implanted groups. From histological study of the animals bearing testosterone implants of either 3 cm or 25 cm in length (Fig.3.28, 32, 35, and 38), many of the interstitial cells were found to regress significantly.

3.3.1 Effect on VEGF-A

In the control animals, VEGF-A immunostaining was localized mainly in the intertubular area within the interstitial cell (Fig.3.28A), although Sertoli cells and vascular smooth muscle cells also contained some immunoreactivity. In animals bearing the testosterone implants, the interstitial cell population regressed considerably and most

of the VEGF-A immunoreactivity was lost from the intertubular area and hence the Leydig cells, and present only in Sertoli cells and vascular smooth muscle cells (Fig.3.28C and E). After receiving a single injection of hCG, there was a partial recovery of the Leydig cell population in the T-implanted animals as indicated by the re-appearance of VEGF-A immunoreactivity in the interstitial cells (Fig.3.28D and F).

In RT-PCR analysis, primers were originally designed to probe for every spliced variants of VEGF-A simultaneously, but it did not detect any changes (Fig.3.30.1 and 2). This might because of the competition between the spliced variants for the same pair of primers. So, an alternative pair of primers was designed (specified for a region that was common to every VEGF-A transcripts) to further check that whether there were effects on VEGF-A transcripts or not (Fig.3.29.1 and 2). The combined data of densitometric quantification taken from six animals per group indicated that VEGF-A dropped in animals bearing implants of either 3 cm or 25 cm (Fig.3.29.1B). Injection of hCG did not induce an increment in VEGF-A transcript in control and 25 cm implant groups but a significant decrement instead (Fig.3.29.2).

Western immunoblotting analysis demonstrated the changes in three different isoforms of VEGF-A, VEGF-A_{188}, VEGF-A_{164} and VEGF-A_{120} (Fig.3.31.1A). The combined data of densitometric quantification taken from six animals per group indicated that VEGF-A_{164} dropped dramatically in the animals bearing testosterone implants of either 3 cm or 25 cm (Fig.3.31.1B). On the contrary, expression of VEGF-A_{188} appeared unaffected while VEGF-A_{120} showed slight decreases which were not statistically significant. These results corresponded to the earlier findings in EDS treatment study demonstrating that Leydig cell was likely the major source of

	Control		Treatment groups			
Testosterone implant	No implant		3 cm		25 cm	
Subcutaneous injection	Saline	hCG	Saline	hCG	Saline	hCG
Paired testis weight (gram)	3.244 ± 0.057	3.562 ± 0.133	2.122 ± 0.261*	2.289 ± 0.245*	2.954 ± 0.112	3.283 ± 0.080
Number of animals	6					

Table 3.1 Effect of Leydig cell suppression followed by hCG stimulation on the testis weight. Leydig cell suppression was achieved by an eight-week treatment with either a low dose (3 cm) or high dose (25 cm) of testosterone-filled subcutaneous implants. At the end of eight weeks, animals were given a single subcutaneous injection of 100 IU hCG and studied 2 days post hCG. Data represent mean ± SE of 6 animals per group. *$P<0.05$, by Kruskal-Wallis one-way ANOVA on ranks followed by Dunnett's test for comparison with the saline-injected control.

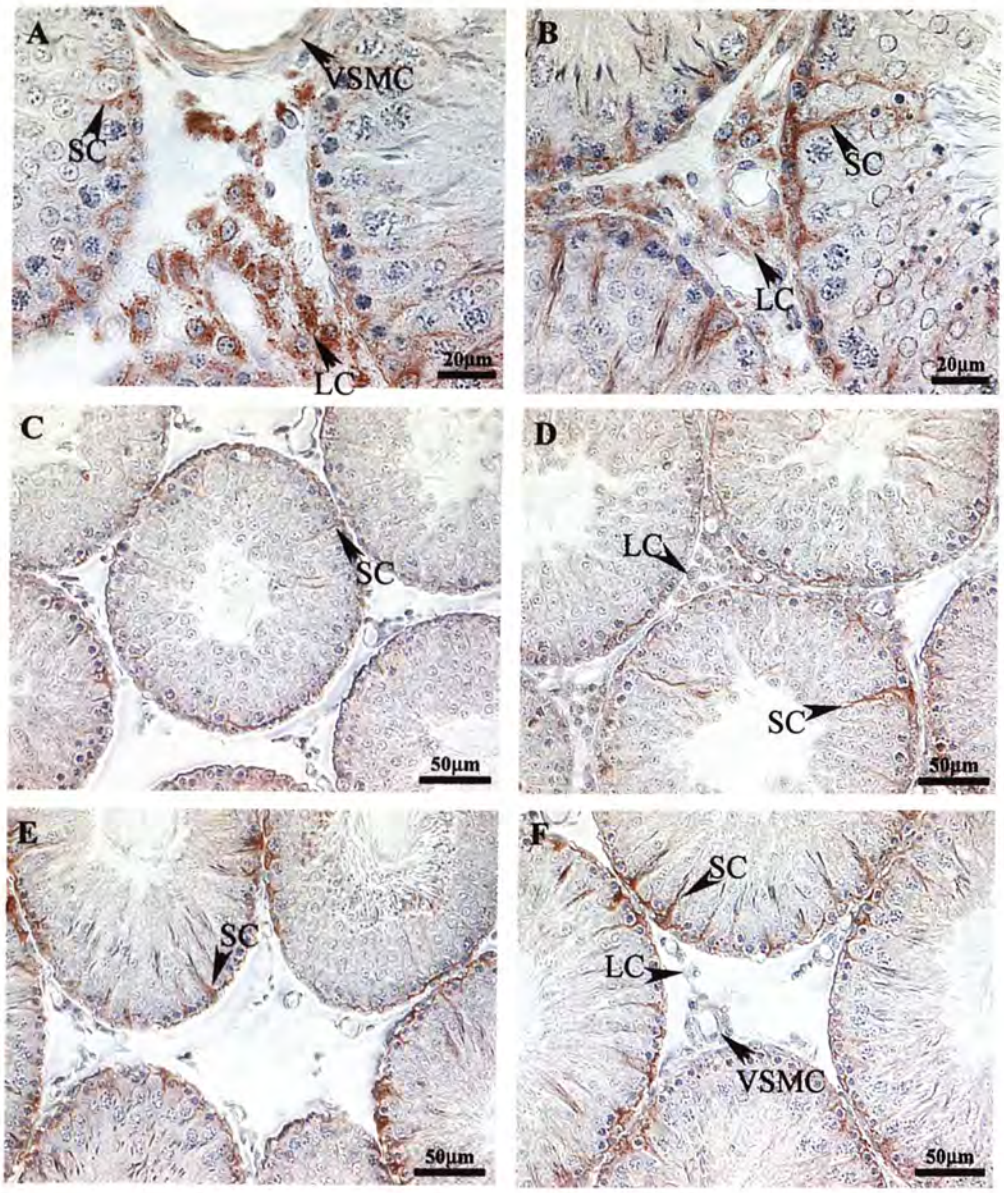


Fig.3.28 Immunohistochemical staining of VEGF-A in the testes of normal adult rats (A & B) and adult rats that received subcutaneous testosterone-filled silastic implants of 3 cm (C & D) or 25 cm (E & F) in length for 8 weeks. Positive VEGFs immunoreactivity was indicated by the red colour and arrows in tissue sections that were counterstained with haematoxylin. Rats in **(B, D & F)** were further injected with a single dose of 100 IU hCG (or saline for **A, C & E**) and studied 2 days post-hCG. Note that many of the interstitial cells regressed in animals bearing testosterone implants of either 3 cm or 25 cm in length **(C & E)**. *Abbreviations stand for: LC: Leydig cells, SC: Sertoli cells and VSMC: vascular smooth muscle cells.*

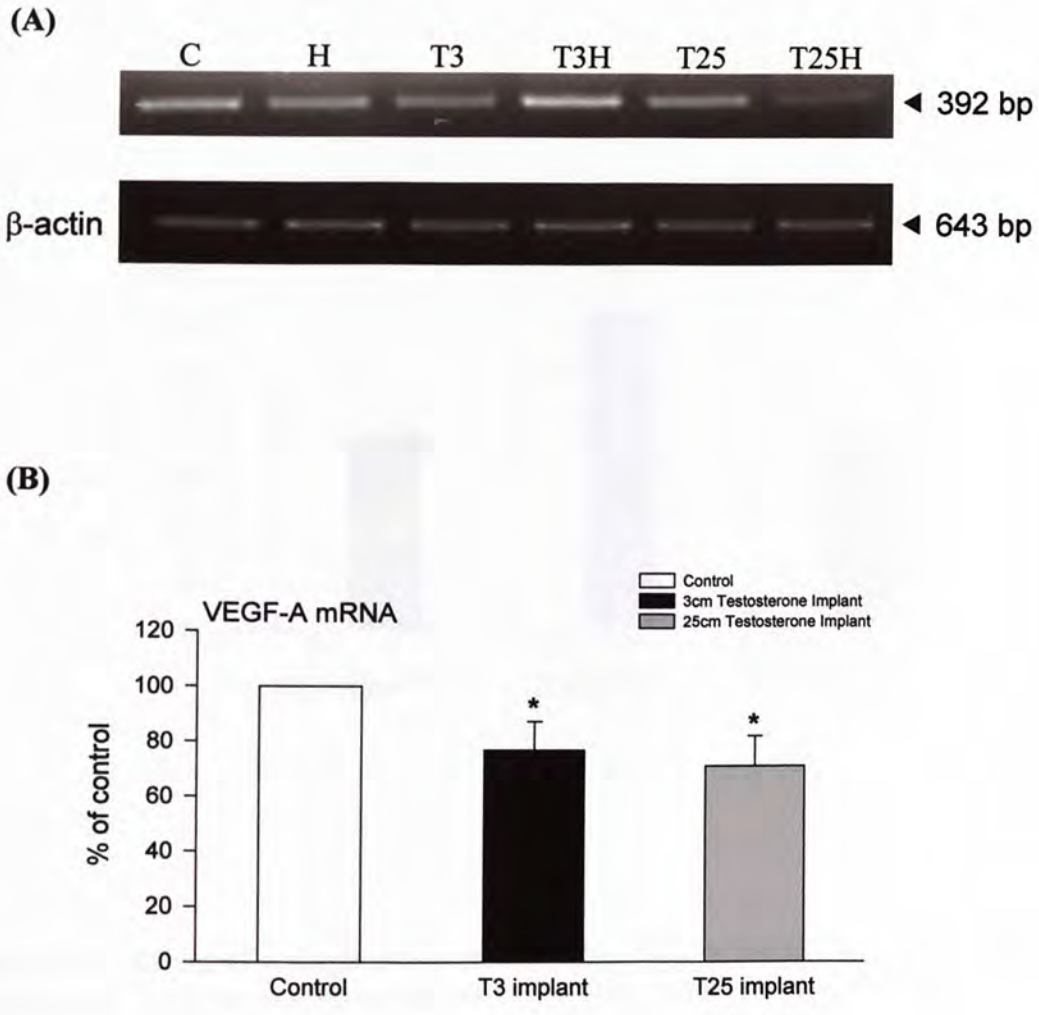


Fig.3.29.1 Effect of Leydig cell suppression by eight-week treatment of subcutaneous testosterone-filled implants on the expression level of the VEGF-A mRNA in the adult rat testis. **(A)** Representative semi-quantitative RT-PCR result showing the effect of treatment on VEGF-A transcript level in the testes. The amplification of β-actin was included as an internal control to adjust the amount of template used. **(B)** Combined data from the densitometric quantification of the semi-quantitative RT-PCR signals of VEGF-A transcript, which were normalized against the control as 100%. Data represent mean ± SE of 6 animals per group. *P<0.05, by Kruskal-Wallis one-way ANOVA on ranks followed by Dunnett's test for comparison with the corresponding control.

Abbreviations stand for: C: control, H: rats received a single injection of 100 IU hCG and examined at 2 days post-injection, T3 & T25: rats received 3 cm or 25 cm subcutaneous testosterone-filled implants for 8 weeks, and T3H & T25H: T-implant bearing rats injected with a single dose of 100 IU hCG and studied 2 days post-hCG injection.

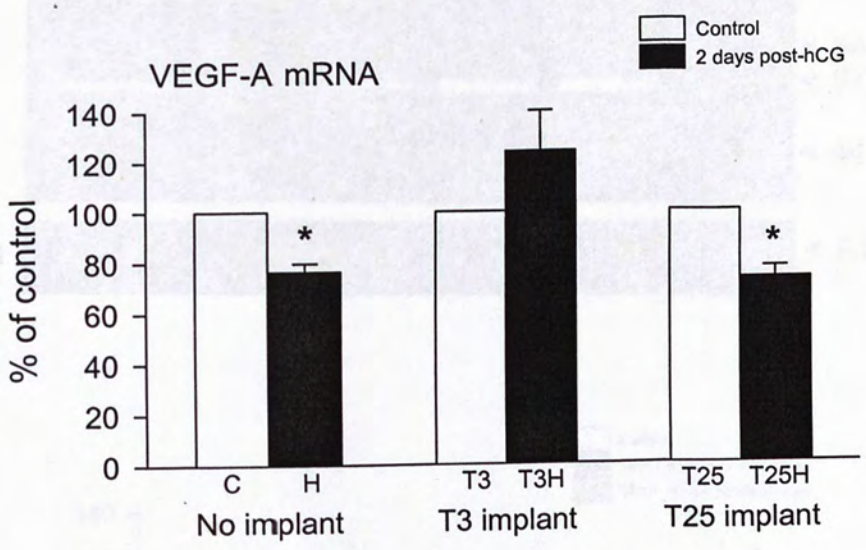


Fig.3.29.2 Effect of a single subcutaneous injection of 100 IU hCG on VEGF-A mRNA expression level in the testes of normal adult rats and rats bearing 3 cm or 25 cm testosterone implants for 8 weeks. Combined data from the densitometric quantification of the semi-quantitative RT-PCR signals of VEGF-A transcript, which were normalized against the control as 100%. Data represent mean ± SE of 6 animals per group. *P<0.05, by Mann-Whitney rank sum test for comparison with the corresponding control.

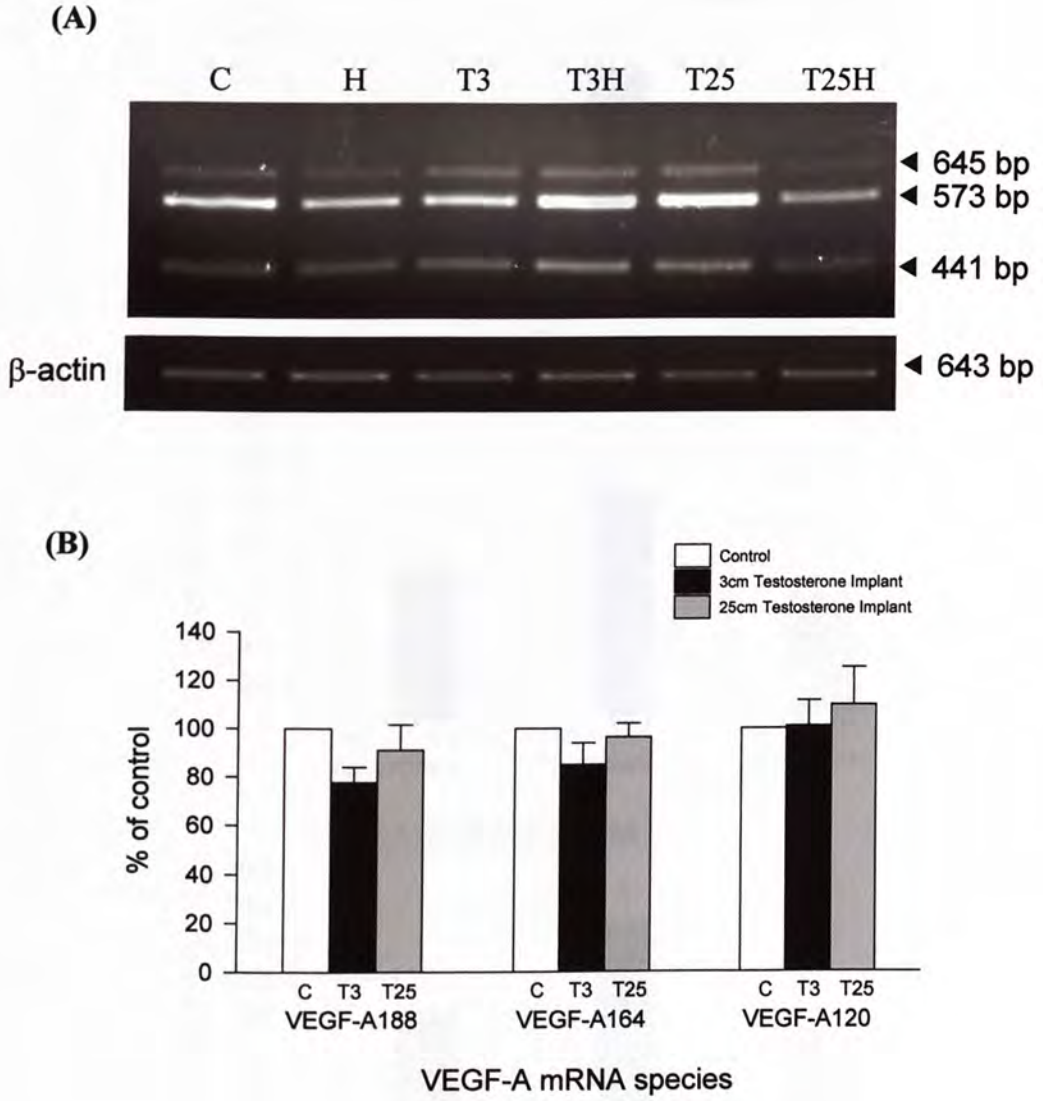


Fig.3.30.1 Effect of Leydig cell suppression by eight-week treatment of subcutaneous testosterone-filled implants on the expression levels of the three VEGF-A mRNA spliced variants expression level in the adult rat testis. **(A)** Representative semi-quantitative RT-PCR result showing the effect of treatment on three VEGF-A spliced variants levels in the testes. The amplification of β-actin was included as an internal control to adjust the amount of template used. **(B)** Combined data from the densitometric quantification of the semi-quantitative RT-PCR signals of VEGF-A spliced variants, which were normalized against the control as 100%. Data represent mean ± SE of 6 animals per group.

Abbreviations stand for: C: control, H: rats received a single injection of 100 IU hCG and examined at 2 days post-injection, T3 & T25: rats received 3 cm or 25 cm subcutaneous testosterone-filled implants for 8 weeks, and T3H & T25H: T-implant bearing rats injected with a single dose of 100 IU hCG and studied 2 days post-hCG injection.

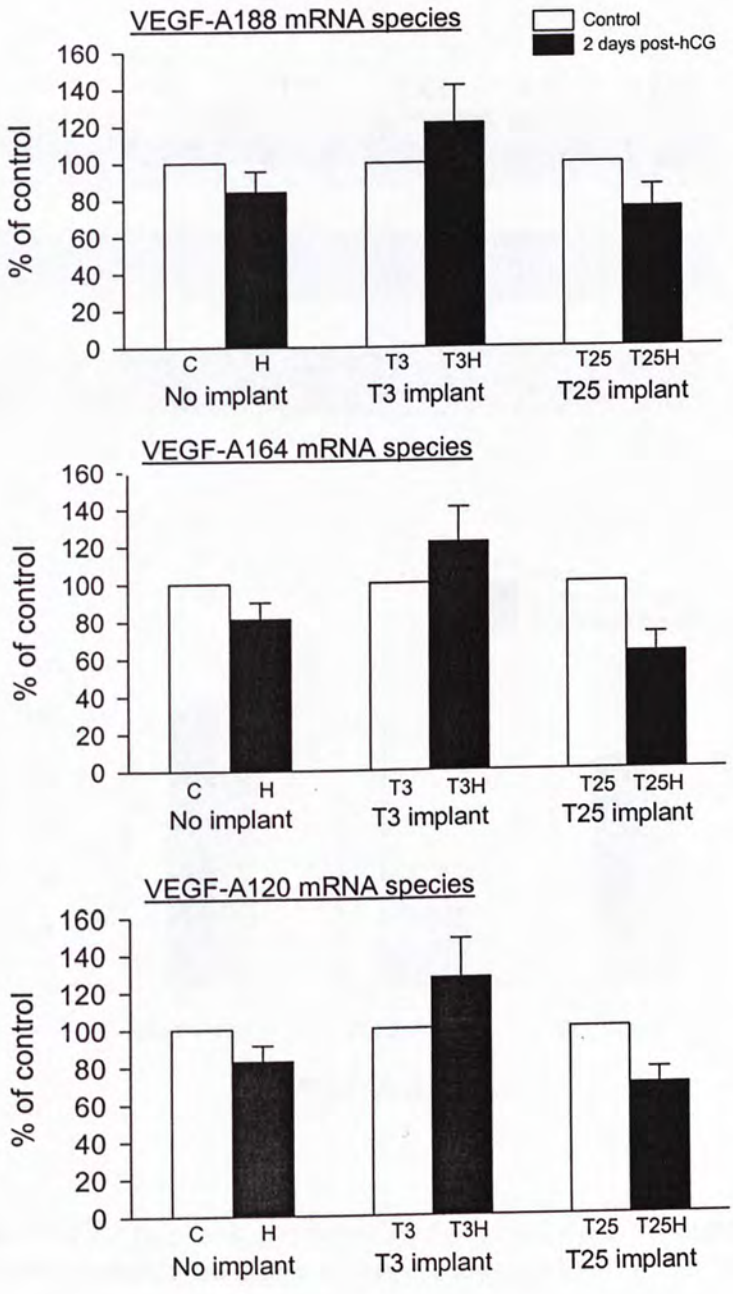


Fig.3.30.2 Effect of a single subcutaneous injection of 100 IU hCG on the expression levels of the three VEGF-A mRNA spliced variants in the testes of normal adult rats and rats bearing 3 cm or 25 cm testosterone implants for 8 weeks. Combined data from the densitometric quantification of the semi-quantitative RT-PCR signals of three VEGF-A spliced variants, which were normalized against the control as 100%. Data represent mean ± SE of 6 animals per group.

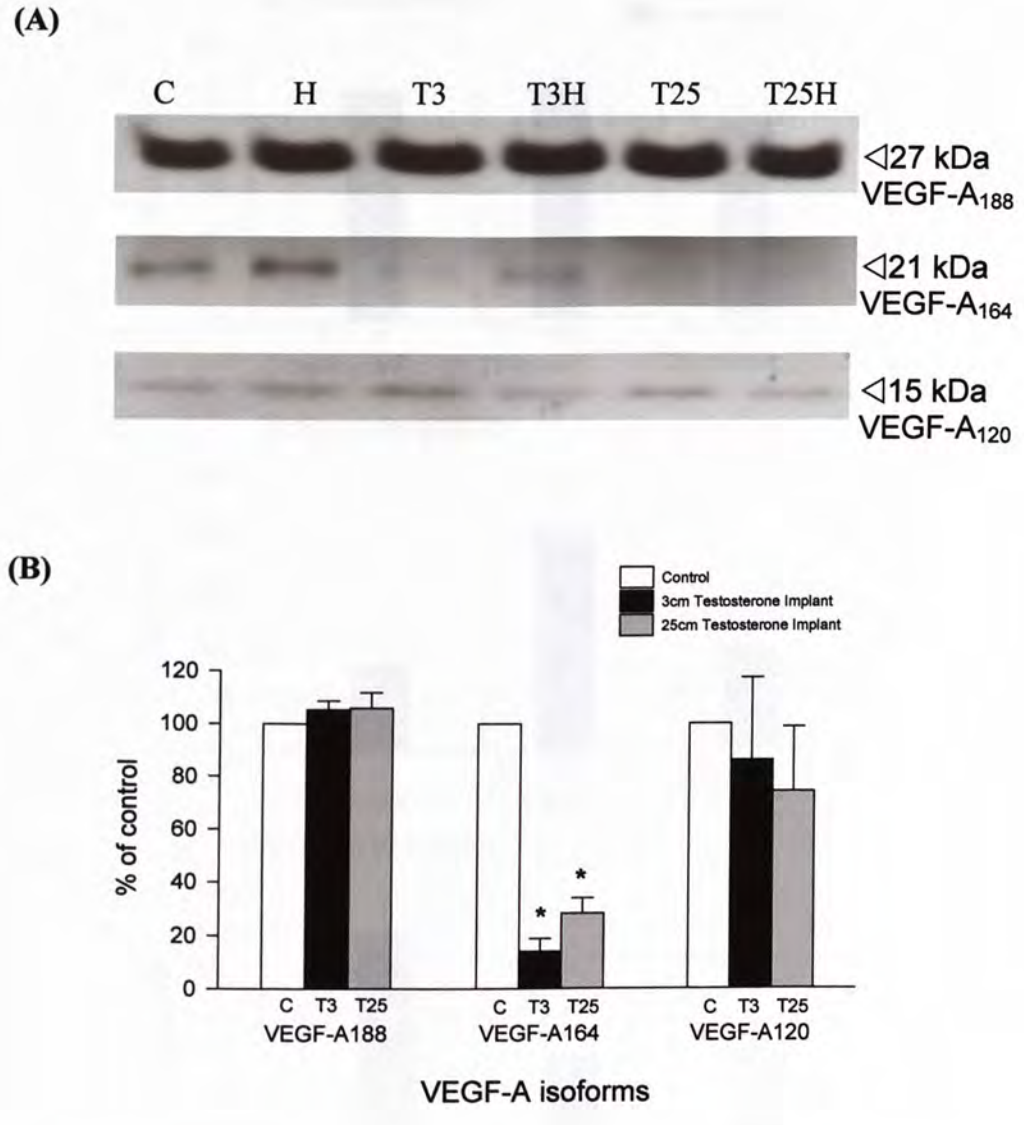


Fig.3.31.1 Effect of Leydig cell suppression by eight-week treatment of subcutaneous testosterone-filled implants on the expression levels of the three VEGF-A isoforms in the adult rat testis. **(A)** A representative Western blot showed the effect of treatment on three VEGF-A isoform levels in the testes. **(B)** Combined data from the densitometric quantification of signal of three VEGF-A isoforms, which were normalized against the control as 100%. Data represent mean ± SE of 6 animals per group. *P<0.05, by Kruskal-Wallis one-way ANOVA on ranks followed by Dunnett's test for comparison with the corresponding control.

Abbreviations stand for: C: control, H: rats received a single injection of 100 IU hCG and examined at 2 days post-injection, T3 & T25: rats received 3 cm or 25 cm subcutaneous testosterone-filled implants for 8 weeks, and T3H & T25H: T-implant bearing rats injected with a single dose of 100 IU hCG and studied 2 days post-hCG injection.

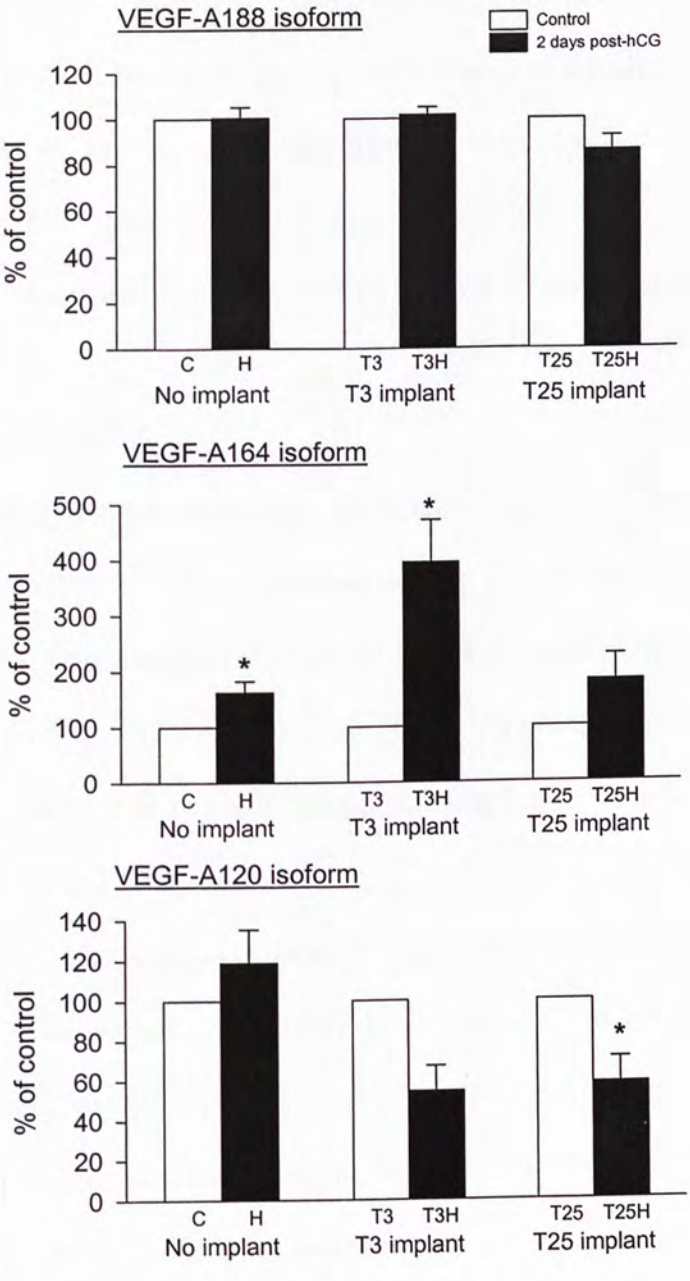


Fig.3.31.2 Effect of a single subcutaneous injection of 100 IU hCG on the expression levels of VEGF-A isoforms in the testes of normal rats and rats bearing either 3 cm or 25 cm testosterone implants for 8 weeks. Combined data from the densitometric quantification of the signals of three VEGF-A isoforms, which were normalized against the control as 100%. Data represent mean ± SE of 6 animals per group. *P<0.05, by Mann-Whitney rank sum test for comparison with the corresponding control.

VEGF-A_{164} isoform (Fig.3.19). Injection of hCG induced increases in VEGF-A_{164} isoform levels in all treatment groups, with statistical significance found in the control and 3 cm implant groups. hCG treatment did not cause significant changes in VEGF-A_{188} levels while those of VEGF-A_{120} showed decreases in the T-implant groups reaching statistical significance in animals bearing 25 cm T-implant (Fig.3.31.2).

3.3.2 Effect on VEGF-B

VEGF-B immunoreactivity was localized mainly in the interstitial cells of the control animals (Fig.3.32A). In animals bearing the testosterone implants, the interstitial cell population regressed noticeably and VEGF-B immunoreactivity was lost from the interstitial area (Fig.3.32C and E). After receiving a single dose of hCG, interstitial cell population recovered in animals bearing the implants, but to a lesser degree in 25 cm implant group (Fig.3.32D and F).

RT-PCR analysis of VEGF-B transcripts demonstrated the changes in two different spliced variants of VEGF-B, VEGF-B_{186} and VEGF-B_{167} (Fig.3.33.1A). Combined data of densitometric quantification taken from six animals per group indicated no significant changes in the levels of VEGF-B_{186} and VEGF-B_{167} transcripts (Fig.3.33.1B). Injection of hCG induced small increases in VEGF-B transcript levels in control animals, reaching statistical significance for VEGF-B_{186} (Fig.3.33.2).

Western immunoblotting analysis demonstrated the changes in two isoforms of VEGF-B, VEGF-B_{186} and VEGF-B_{167} (Fig.3.34.1A). The combined data of densitometric quantification taken from six animals per group indicated that VEGF-B isoforms dropped generally in two implant groups while only VEGF-B_{167} dropped to a significant level in the animals bearing testosterone implants of 25 cm (Fig.3.34.1B).

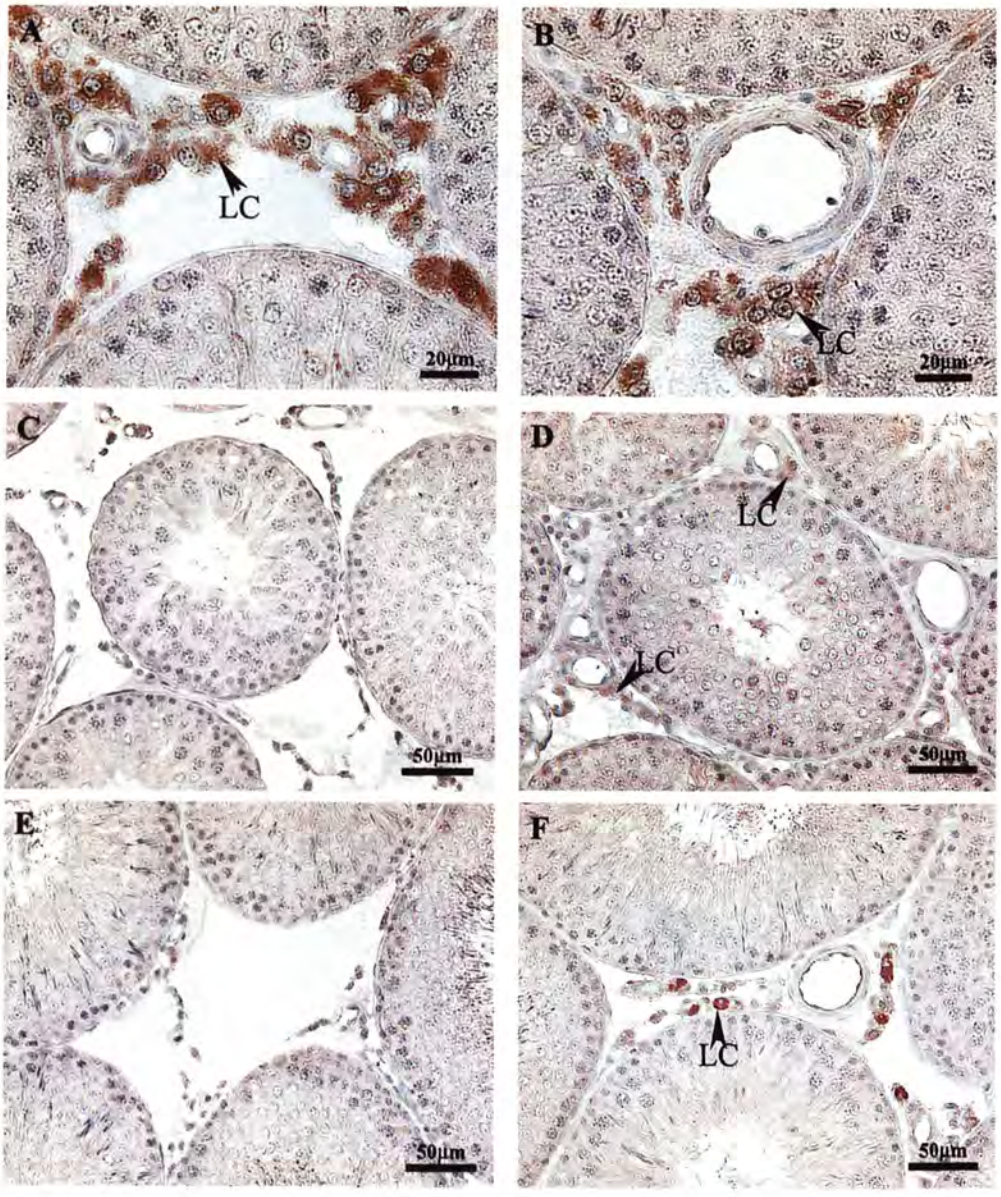


Fig.3.32 Immunohistochemical staining of VEGF-B in the testes of normal adult rats (A & B) and adult rats that received subcutaneous testosterone-filled silastic implants of 3 cm (C & D) or 25 cm (E & F) in length for 8 weeks. Positive VEGFs immunoreactivity was indicated by the red colour and arrows in tissue sections that were counterstained with haematoxylin. Rats in **(B, D & F)** were further injected with a single dose of 100 IU hCG (or saline for **A, C & E**) and studied 2 days post-hCG. Note that many of the interstitial cells regressed in animals bearing testosterone implants of either 3 cm or 25 cm in length **(C & E)**. *Abbreviations stand for: LC: Leydig cells, SC: Sertoli cells and VSMC: vascular smooth muscle cells.*

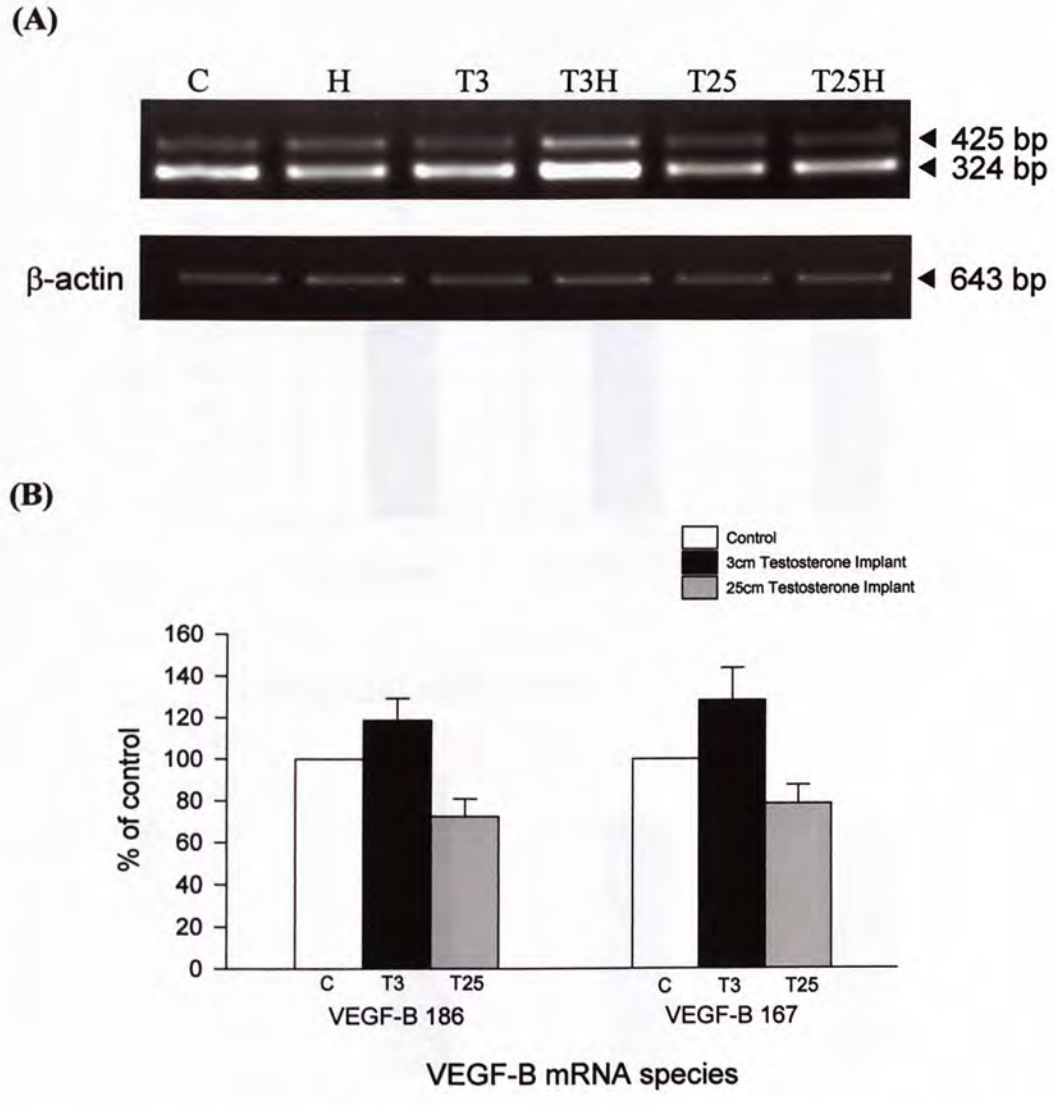


Fig.3.33.1 Effect of Leydig cell suppression by eight-week treatment of subcutaneous testosterone-filled implants on two VEGF-B mRNA spliced variants expression level in the adult rat testis. (A) Representative semi-quantitative RT-PCR result showing the effect of treatment on two VEGF-B transcripts level in the testes. The amplification of β-actin was included as an internal control to adjust the amount of template used. **(B)** Combined data from the densitometric quantification of the semi-quantitative RT-PCR signals of VEGF-B transcripts, which were normalized against the control as 100%. Data represent mean ± SE of 6 animals per group.

Abbreviations stand for: C: control, H: rats received a single injection of 100 IU hCG and examined at 2 days post-injection, T3 & T25: rats received 3 cm or 25 cm subcutaneous testosterone-filled implants for 8 weeks, and T3H & T25H: T-implant bearing rats injected with a single dose of 100 IU hCG and studied 2 days post-hCG injection.

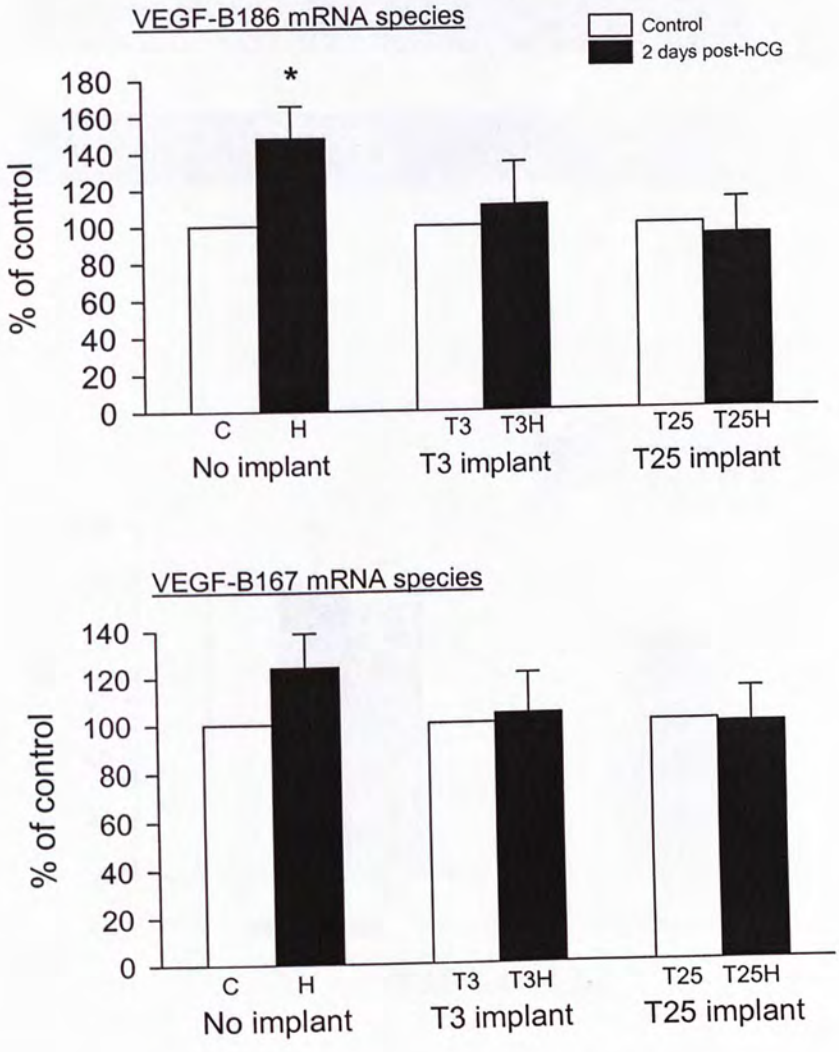


Fig.3.33.2 Effect of a single subcutaneous injection of 100 IU hCG on the expression levels of the two spliced variants of VEGF-B mRNA in the testes of normal adult rats and rats bearing 3 cm or 25 cm testosterone implants for 8 weeks. Combined data from the densitometric quantification of the semi-quantitative RT-PCR signals of two VEGF-B spliced variants, which were normalized against the control as 100%. Data represent mean ± SE of 6 animals per group. *P<0.05, by Mann-Whitney rank sum test for comparison with the corresponding control.

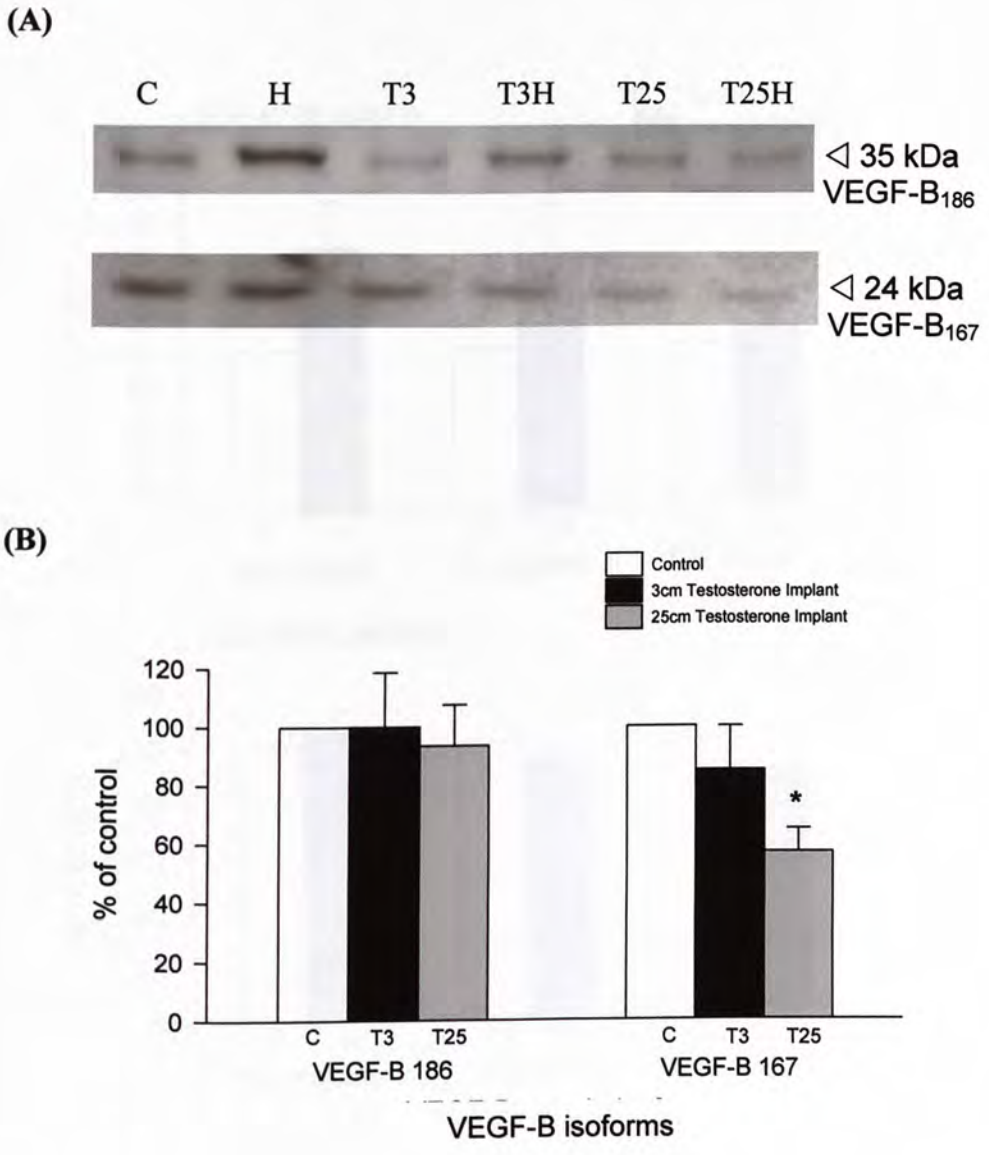


Fig.3.34.1 Effect of Leydig cell suppression by eight-week treatment of subcutaneous testosterone-filled implants on the expression levels of the two VEGF-B isoforms in the adult rat testis. **(A)** A representative Western blot showed the effect of treatment on two VEGF-B isoform levels in the testes. **(B)** Combined data from the densitometric quantification of signal of two VEGF-B isoforms, which were normalized against the control as 100%. Data represent mean ± SE of 6 animals per group. *P<0.05, by Kruskal-Wallis one-way ANOVA on ranks followed by Dunnett's test for comparison with the corresponding control.

Abbreviations stand for: C: control, H: rats received a single injection of 100 IU hCG and examined at 2 days post-injection, T3 & T25: rats received 3 cm or 25 cm subcutaneous testosterone-filled implants for 8 weeks, and T3H & T25H: T-implant bearing rats injected with a single dose of 100 IU hCG and studied 2 days post-hCG injection.

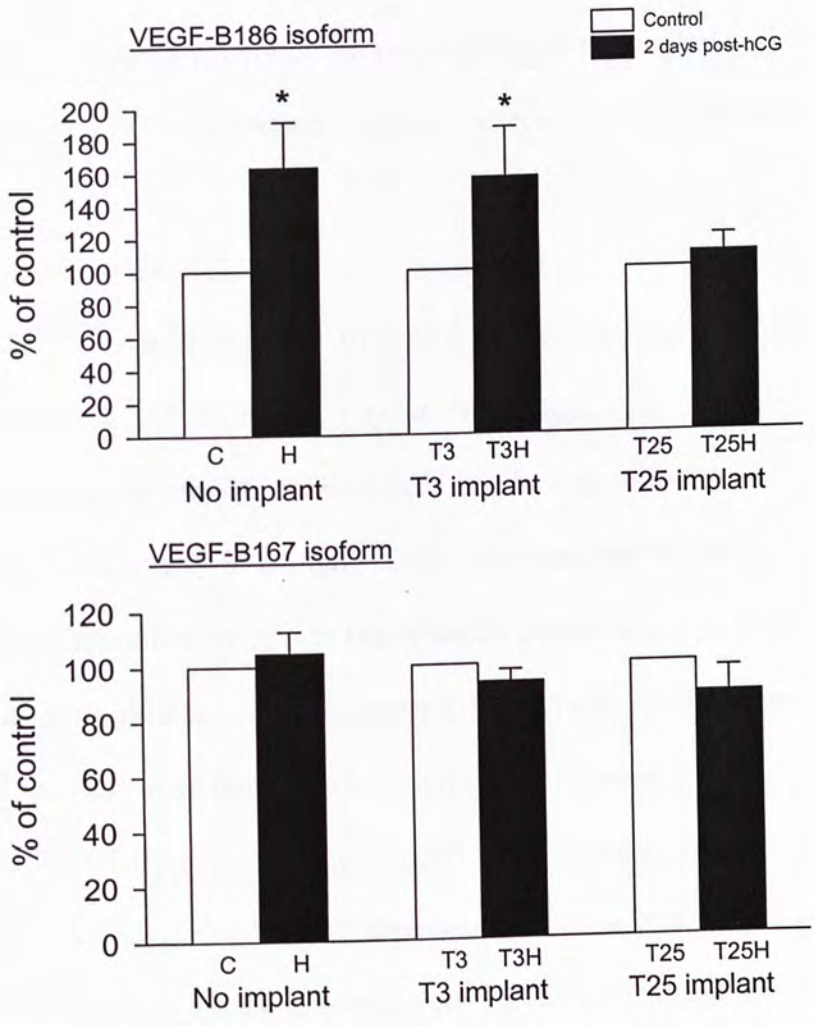


Fig.3.34.2 Effect of a single subcutaneous injection of 100 IU hCG on the expression levels of VEGF-B isoforms in the testes of normal adult rats and rats bearing either 3 cm or 25 cm testosterone implants for 8 weeks. Combined data from the densitometric quantification of the signals of two VEGF-B isoforms, which were normalized against the control as 100%. Data represent mean ± SE of 6 animals per group. *P<0.05, by Mann-Whitney rank sum test for comparison with the corresponding control.

On the other hand, expression of VEGF-B_{186} was little affected by the suppression of Leydig cell function by T-implants. Injection of hCG caused an overall significant increase in VEGF-B_{186} peptide levels, but only in the control and 3 cm implant groups (Fig.3.34.2). VEGF-B_{167} seemed not to be affected by hCG treatment.

3.3.3 Effect on VEGF-C

In the control animals, VEGF-C immunostaining was localized chiefly in the interstitial cells and hence the Leydig cells (Fig.3.35A). Immunoreactivity in Sertoli cells and vascular smooth muscle cells was not observed. Interstitial cell population regressed considerably in animals bearing the testosterone implants and nearly all of the VEGF-C immunoreactivity was lost from the intertubular area (Fig.3.35C and E). After receiving a single dose of hCG, interstitial cell population in animals bearing implants showed recovery with the return of VEGF-C immunoreactivity (Fig.3.35D and F).

RT-PCR analysis demonstrated the changes in VEGF-C mRNA levels (Fig.3.36.1A). Combined data of densitometric quantification taken from six animals per group demonstrated a decline of expression of VEGF-C transcripts in animals bearing 25 cm implant (Fig.3.36.1B). Injection of hCG generally increased VEGF-C transcripts expression in control and treatment groups but a significant increase was only noted in the 25 cm implant group (Fig.3.36.2).

Western immunoblotting analysis demonstrated a marked decrease in VEGF-C peptide levels (Fig.3.37.1A) in the testes of animals bearing testosterone implants of either 3 cm or 25 cm (indicated by the combined data of densitometric quantification taken from six animals per group) (Fig.3.37.1B). These results correlated to the earlier

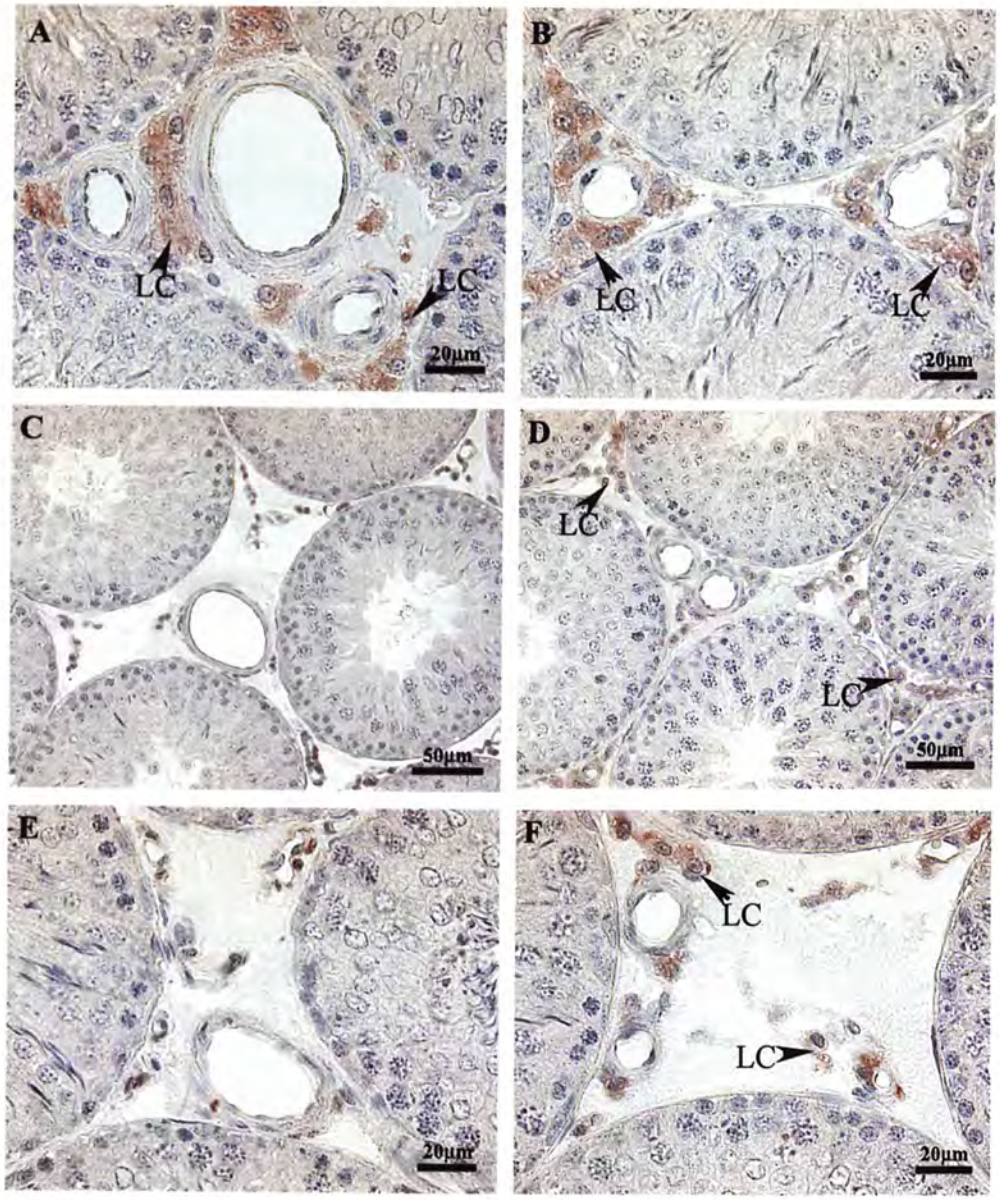


Fig.3.35 Immunohistochemical staining of VEGF-C in the testes of normal adult rats (A & B) and adult rats that received subcutaneous testosterone-filled silastic implants of 3 cm (C & D) or 25 cm (E & F) in length for 8 weeks. Positive VEGFs immunoreactivity was indicated by the red colour and arrows in tissue sections that were counterstained with haematoxylin. Rats in **(B, D & F)** were further injected with a single dose of 100 IU hCG (or saline for **A, C & E**) and studied 2 days post-hCG. Note that many of the interstitial cells regressed in animals bearing testosterone implants of either 3 cm or 25 cm in length **(C & E)**. *Abbreviations stand for: LC: Leydig cells, SC: Sertoli cells and VSMC: vascular smooth muscle cells.*

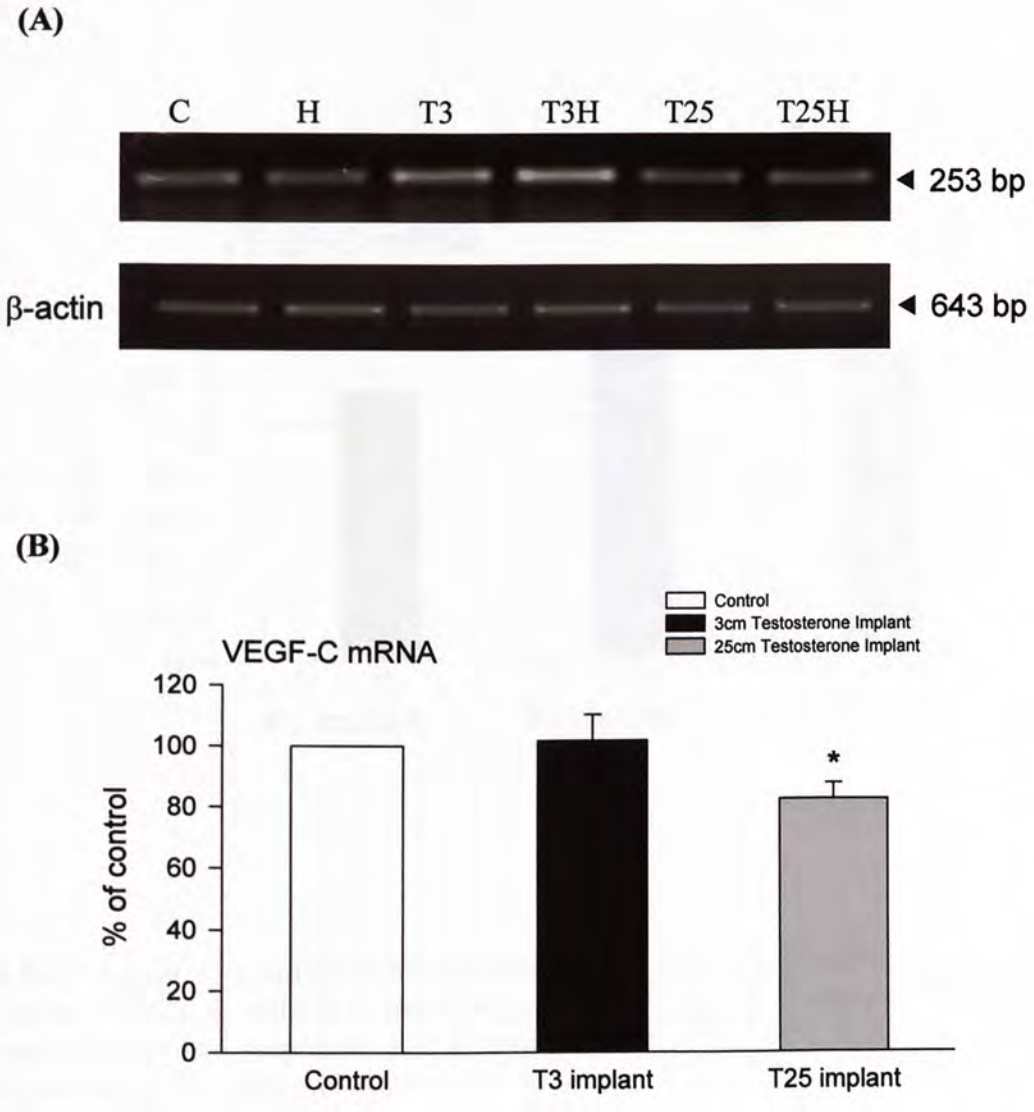


Fig.3.36.1 Effect of Leydig cell suppression by eight-week treatment of subcutaneous testosterone-filled implants on the expression level of the VEGF-C mRNA in the adult rat testis. **(A)** Representative semi-quantitative RT-PCR result showing the effect of treatment on VEGF-C transcript level in the testes. The amplification of β-actin was included as an internal control to adjust the amount of template used. **(B)** Combined data from the densitometric quantification of the semi-quantitative RT-PCR signals of VEGF-C transcript, which was normalized against the control as 100%. Data represent mean ± SE of 6 animals per group. *P<0.05, by Kruskal-Wallis one-way ANOVA on ranks followed by Dunnett's test for comparison with the corresponding control.
Abbreviations stand for: C: control, H: rats received a single injection of 100 IU hCG and examined at 2 days post-injection, T3 & T25: rats received 3 cm or 25 cm subcutaneous testosterone-filled implants for 8 weeks, and T3H & T25H: T-implant bearing rats injected with a single dose of 100 IU hCG and studied 2 days post-hCG injection.

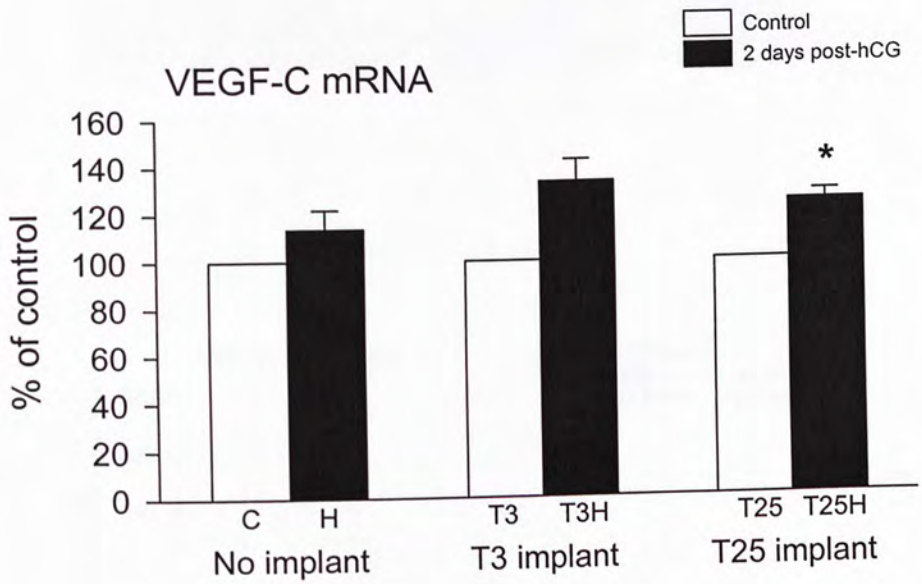
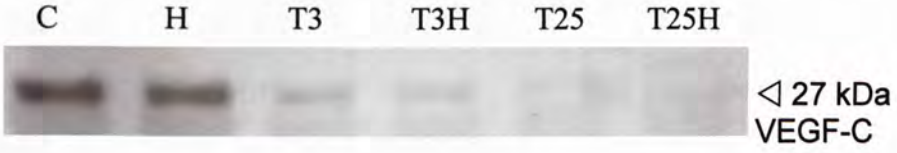


Fig.3.36.2 Effect of a single subcutaneous injection of 100 IU hCG on the expression level of the VEGF-C mRNA in the testes of normal adult rats and rats bearing 3 cm or 25 cm testosterone implants for 8 weeks. Combined data from the densitometric quantification of the semi-quantitative RT-PCR signals of VEGF-C transcript, which were normalized against the control as 100%. Data represent mean ± SE of 6 animals per group. *P<0.05, by Mann-Whitney rank sum test for comparison with the corresponding control.

(A)



(B)

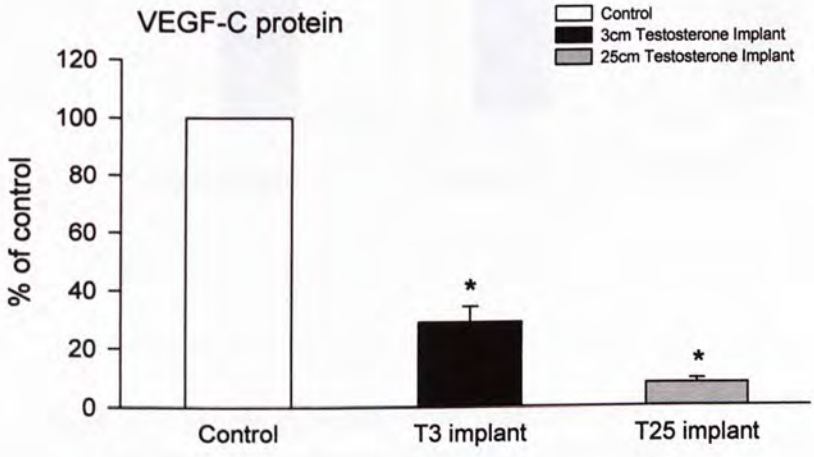


Fig.3.37.1 Effect of Leydig cell suppression by eight-week treatment of subcutaneous testosterone-filled implants on the expression level of the VEGF-C protein in the adult rat testis. **(A)** A representative Western blot showed the effect of treatment on VEGF-C protein level in the testes. **(B)** Combined data from the densitometric quantification of signal of VEGF-C, which were normalized against the control as 100%. Data represent mean ± SE of 6 animals per group. *P<0.05, by Kruskal-Wallis one-way ANOVA on ranks followed by Dunnett's test for comparison with the corresponding control.
Abbreviations stand for: C: control, H: rats received a single injection of 100 IU hCG and examined at 2 days post-injection, T3 & T25: rats received 3 cm or 25 cm subcutaneous testosterone-filled implants for 8 weeks, and T3H & T25H: T-implant bearing rats injected with a single dose of 100 IU hCG and studied 2 days post-hCG injection.

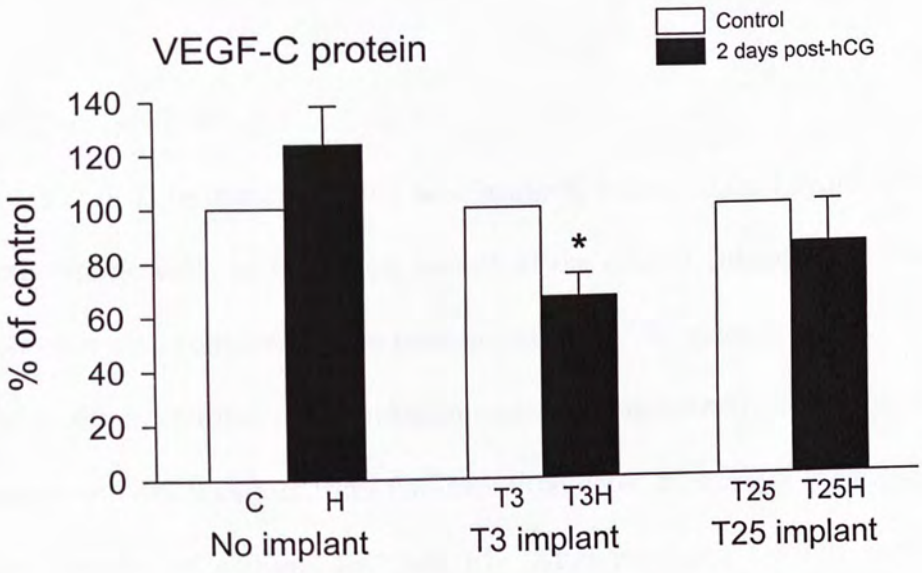


Fig.3.37.2 Effect of a single subcutaneous injection of 100 IU hCG on the expression level of VEGF-C protein in the testes of normal adult rats and rats bearing either 3 cm or 25 cm testosterone implants for 8 weeks. Combined data from the densitometric quantification of the signals of VEGF-C, which were normalized against the control as 100%. Data represent mean ± SE of 6 animals per group. *P<0.05, by Mann-Whitney rank sum test for comparison with the corresponding control.

findings in EDS treatment study that Leydig cell was proposed to be the major source of VEGF-C peptide (Fig.3.23). Injection of hCG caused inconsistent changes in VEGF-C expression with a significant decrease in the 3 cm implant group (Fig.3.37.2).

3.3.4 Effect on VEGF-D

VEGF-D immunoreactivity was localized mainly in the Leydig cells and vascular smooth muscle cells of the blood vessels of the control animals (Fig.3.38A), although Sertoli cells also contained some immunoreactivity. In animals bearing the testosterone implants, the interstitial cell population regressed considerably and most of the VEGF-D immunoreactivity was lost from the interstitial cells, except the one present in vascular smooth muscle cells (Fig.3.38C and E). After receiving a single injection of hCG, regression of interstitial cell population was recovered in animals bearing the implants, but such recovery was to a lesser extent in 25 cm implant group (Fig.3.38D and F), and in comparison, the expression of VEGF-D in Sertoli cells apparently became more obvious.

RT-PCR analysis demonstrated the changes in of VEGF-D transcripts (Fig.3.39.1A). Combined data of densitometric quantification taken from six animals per group demonstrated a tendency of decline of expression of VEGF-D transcripts in the testosterone implanted groups despite of statistical insignificance (Fig.3.39.1B). Injection of hCG generally induced an increment in VEGF-D transcripts in control and treatment groups, and among them, a significant increase was only noted in the 3 cm and 25 cm implant groups (Fig.3.39.2).

Western immunoblotting analysis demonstrated the changes in three isoforms of VEGF-D (Fig.3.40.1A). The combined data of densitometric quantification taken from six animals per group indicated that the 16 kDa peptide increased significantly in the

animals bearing testosterone implants of either 3 cm or 25 cm (Fig.3.40.1B). However, there were no significance both in the 37 kDa and 23 kDa peptides. These results could correlate with the earlier findings in EDS treatment study that the removal of androgen caused the 16 kDa peptide level to rise (Fig.3.25). Injection of hCG caused a general increasing trend in VEGF-D isoforms, but statistical significance were only found in the 23 kDa peptide of the control group and 16 kDa peptide of the 3 cm implant group (Fig.3.40.2).

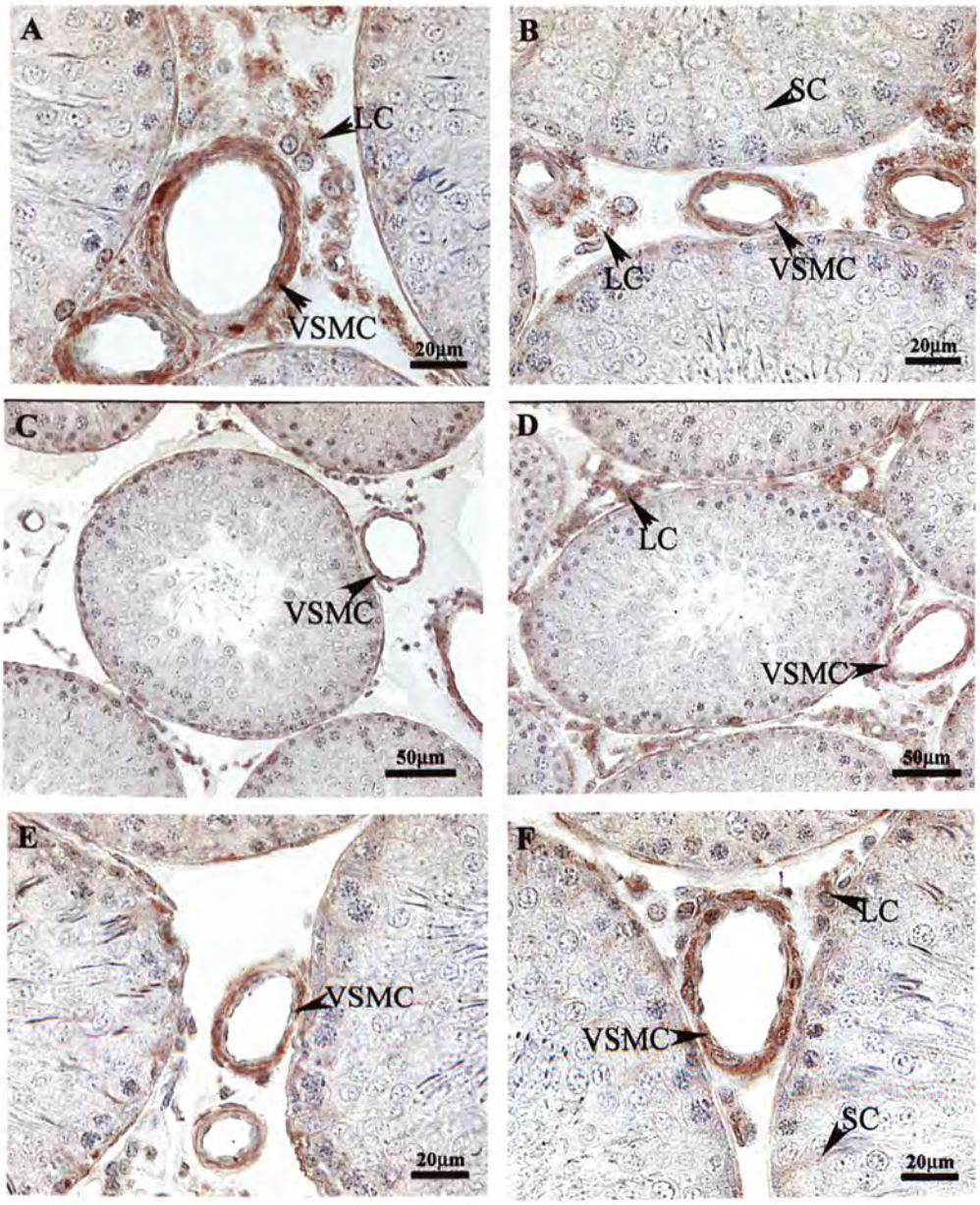


Fig.3.38 Immunohistochemical staining of VEGF-D in the testes of normal adult rats (A & B) and adult rats that received subcutaneous testosterone-filled silastic implants of 3 cm (C & D) or 25 cm (E & F) in length for 8 weeks. Positive VEGFs immunoreactivity was indicated by the red colour and arrows in tissue sections that were counterstained with haematoxylin. Rats in **(B, D & F)** were further injected with a single dose of 100 IU hCG (or saline for **A, C & E**) and studied 2 days post-hCG. Note that many of the interstitial cells regressed in animals bearing testosterone implants of either 3 cm or 25 cm in length **(C & E)**. *Abbreviations stand for: LC: Leydig cells, SC: Sertoli cells and VSMC: vascular smooth muscle cells of the blood vessels.*

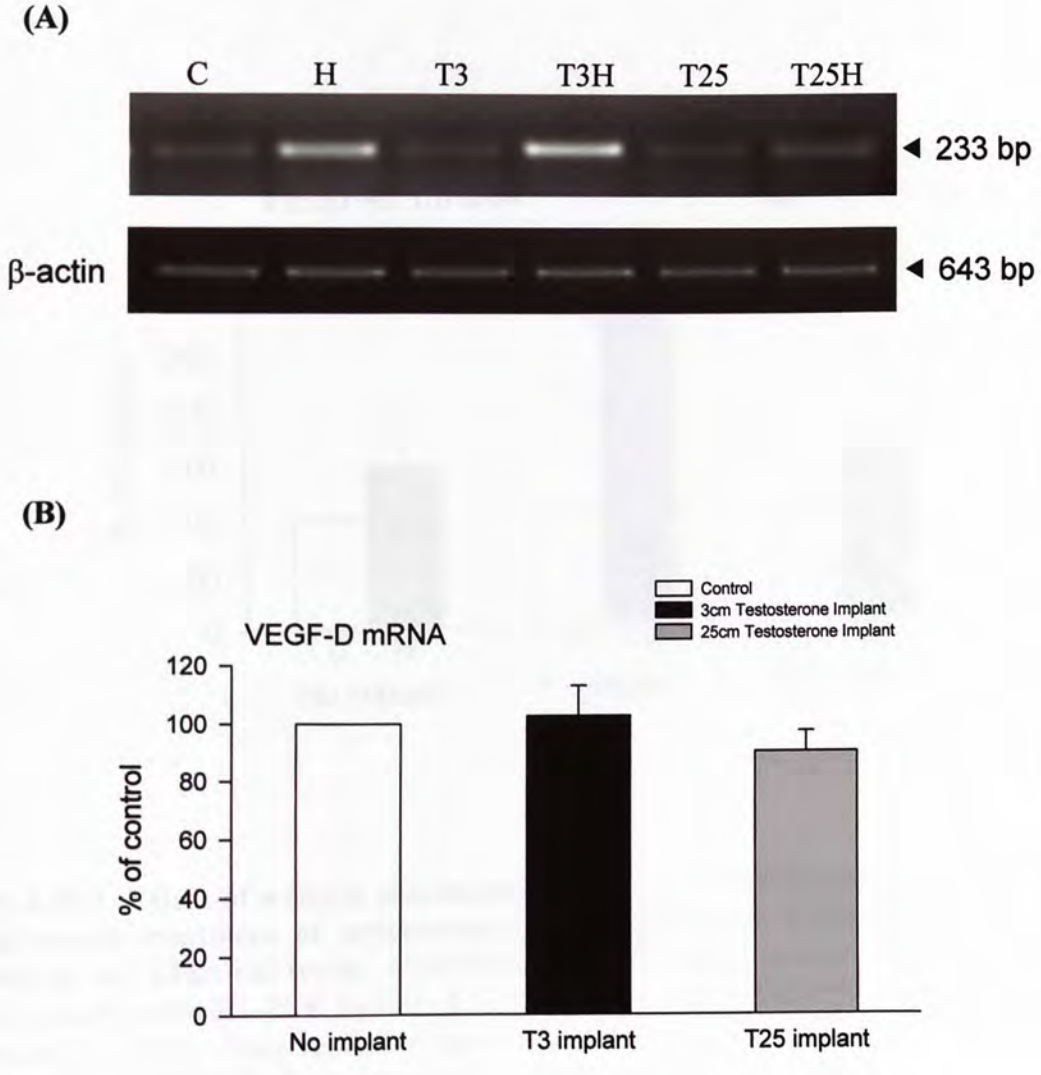


Fig.3.39.1 Effect of Leydig cell suppression by eight-week treatment of subcutaneous testosterone-filled implants on VEGF-D mRNA expression level in the adult rat testis. **(A)** Representative semi-quantitative RT-PCR result showing the effect of treatment on VEGF-D transcript level in the testes. The amplification of β-actin was included as an internal control to adjust the amount of template used. **(B)** Combined data from the densitometric quantification of the semi-quantitative RT-PCR signals of VEGF-D transcript, which was normalized against the control as 100%. Data represent mean ± SE of 6 animals per group.

Abbreviations stand for: C: control, H: rats received a single injection of 100 IU hCG and examined at 2 days post-injection, T3 & T25: rats received 3 cm or 25 cm subcutaneous testosterone-filled implants for 8 weeks, and T3H & T25H: T-implant bearing rats injected with a single dose of 100 IU hCG and studied 2 days post-hCG injection.

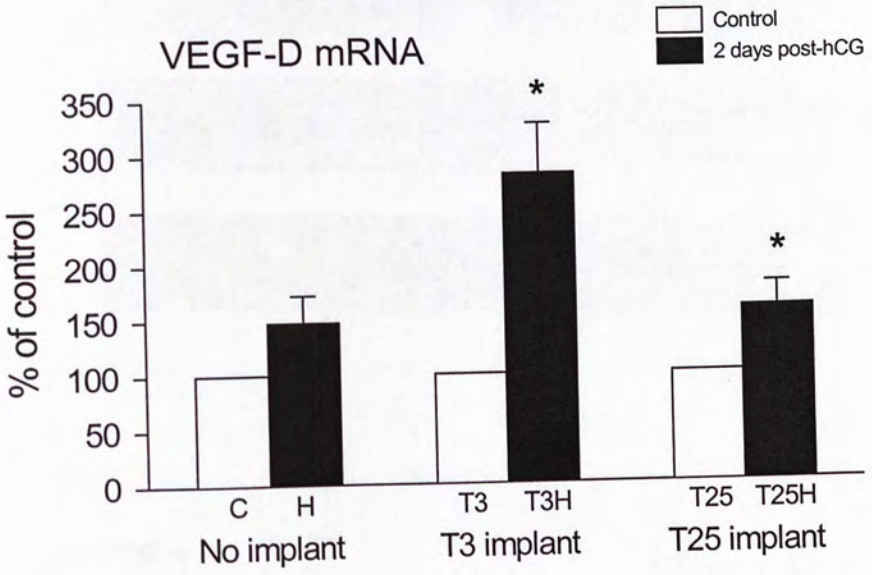


Fig.3.39.2 Effect of a single subcutaneous injection of 100 IU hCG for two days to the eight-week treatment of testosterone-filled implants on VEGF-D mRNA expression level in the adult rat testis. Combined data from the densitometric quantification of the semi-quantitative RT-PCR signals of VEGF-D transcript, which were normalized against the control as 100%. Data represent mean ± SE of 6 animals per group. *P<0.05, by Mann-Whitney rank sum test for comparison with the corresponding control.

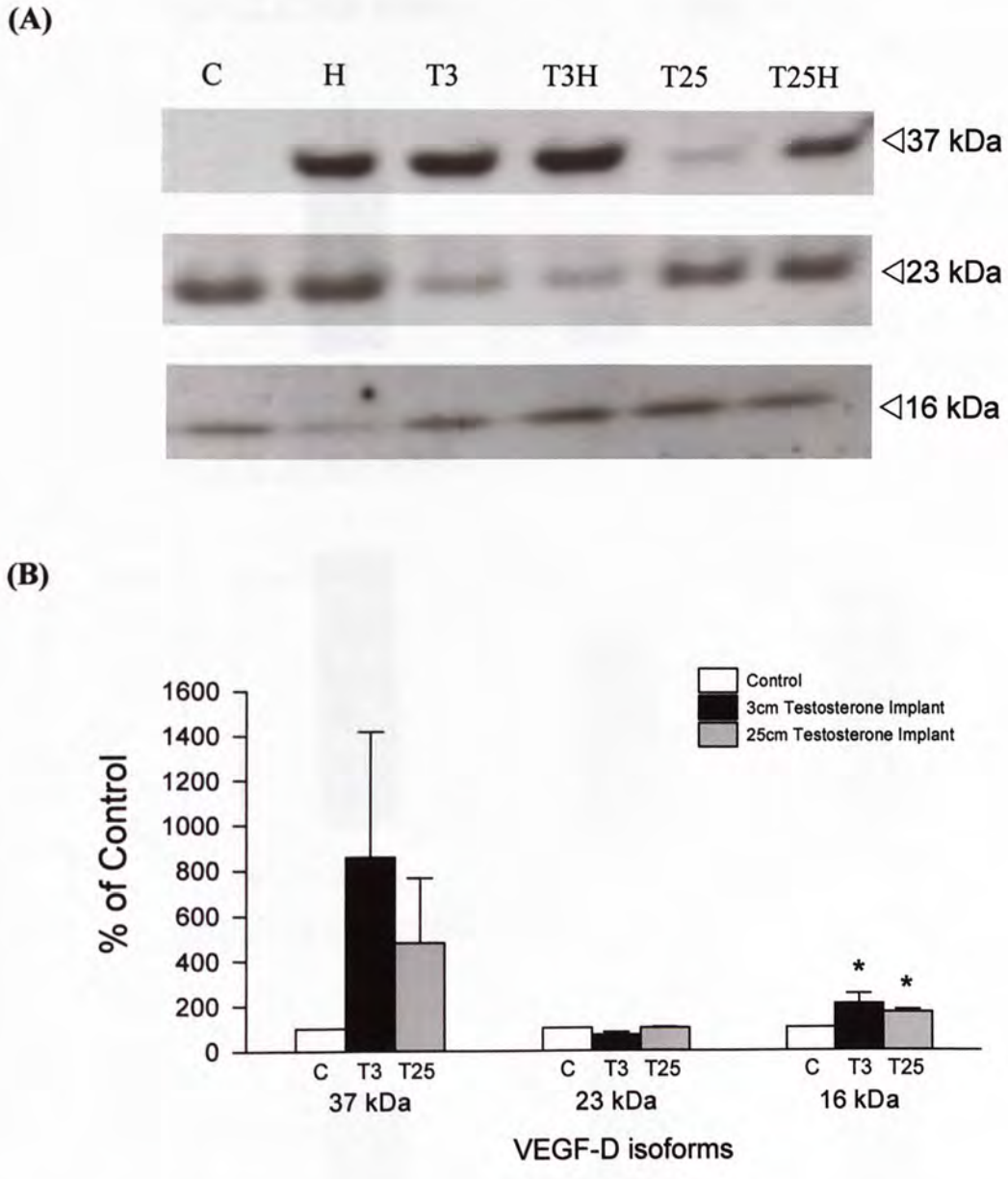


Fig.3.40.1 Effect of Leydig cell suppression by eight-week treatment of subcutaneous testosterone-filled implants on VEGF-D protein isoforms expression level in the adult rat testis. **(A)** A representative Western blot showed the effect of treatment on three VEGF-D isoforms level in the testes. **(B)** Combined data from the densitometric quantification of signal of three VEGF-D isoforms, which were normalized against the control as 100%. Data represent mean ± SE of 6 animals per group. *P<0.05, by Kruskal-Wallis one-way ANOVA on ranks followed by Dunnett's test for comparison with the corresponding control.
Abbreviations stand for: C: control, H: rats received a single injection of 100 IU hCG and examined at 2 days post-injection, T3 & T25: rats received 3 cm or 25 cm subcutaneous testosterone-filled implants for 8 weeks, and T3H & T25H: T-implant bearing rats injected with a single dose of 100 IU hCG and studied 2 days post-hCG injection.

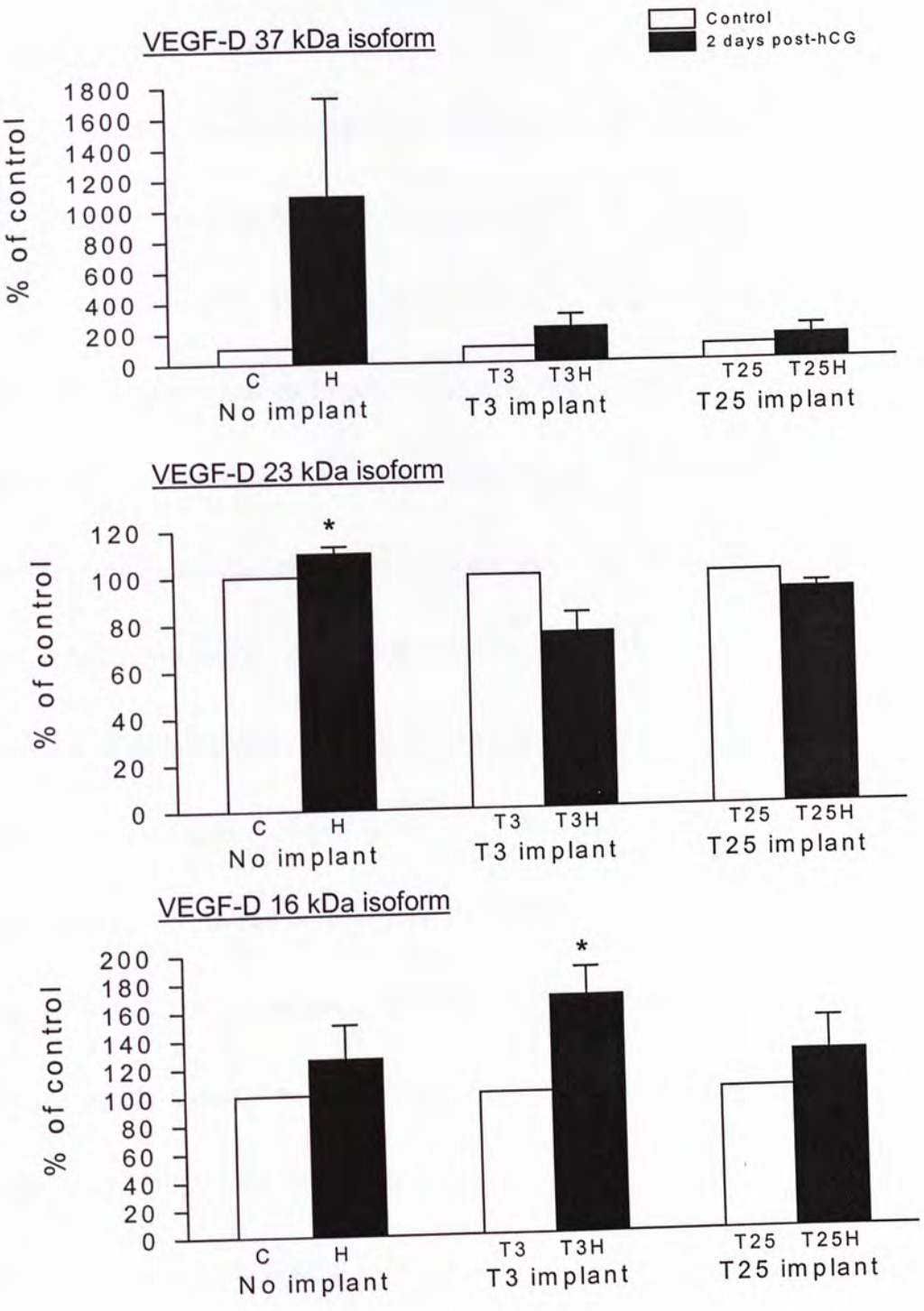


Fig.3.40.2 Effect of a single subcutaneous injection of 100 IU hCG for two days to the eight-week treatment of testosterone-filled implants on VEGF-D protein isoforms expression level in the adult rat testis. Combined data from the densitometric quantification of the signals of three VEGF-D isoforms, which were normalized against the control as 100%. Data represent mean ± SE of 6 animals per group. *P<0.05, by Mann-Whitney rank sum test for comparison with the corresponding control.

4. Discussion

Early studies have revealed that VEGF-A is synthesized in the testis and predominantly associated with the Leydig cells (Au *et al.*, 1997; Collin & Bergh, 1996; Korpelainen *et al.*, 1998; Shweiki *et al.*, 1993; Wan *et al.*, 1996), however the control of its expression is largely unknown. Four other members of the VEGF family, PlGF, VEGF-B, VEGF-C and VEGF-D, have been then identified to share structural and functional similarity in expression patterns, receptor specificity and cellular function (Joukov *et al.*, 1996; Lee *et al.*, 1996; Leung *et al.*, 1989; Li & Eriksson, 2001; Maglione *et al.*, 1991; Olofsson *et al.*, 1996a; Olofsson *et al.*, 1999). Furthermore, expression of their receptors (VEGFR-1, VEGFR-2 and VEGFR-3) has been identified in rat and human tissues (Galland *et al.*, 1993; Pajusola *et al.*, 1993; Shibuya *et al.*, 1990; Sandner *et al.*, 1997; Terman *et al.*, 1991; Wen *et al.*, 1998; Yamane *et al.*, 1994). In the present study, we demonstrated the expression and localization of VEGF-A, PlGF, VEGF-B, VEGF-C and VEGF-D in the rat testis and they expressed in both Leydig cells and Sertoli cells. These findings would suggest that the VEGF family members (VEGFs) may have a complex interaction with each other in autocrine or paracrine manner in the testis.

In the present study, apart from VEGF-A, four other members of the VEGF family (PlGF, VEGF-B, VEGF-C and VEGF-D) have been demonstrated to be

present in the testis and they are immunolocalized mainly to Leydig cells. Using RT-PCR and Western immunoblotting, these peptides were also found to be expressed in Sertoli cells.

Since the four members of the VEGF family (PlGF, VEGF-B, VEGF-C and VEGF-D) have been discovered for about a decade only, very few studies have been done on rat tissues. As a result, deduction of the molecular weight of these peptides in rat would largely depend on the findings from human and mouse, and the specificity of the purchased antibodies.

The molecular weight of PlGF as revealed by Western blotting in the present study was 27 kDa. This finding is close to the study by DiPalma *et al.* (1996), where the molecular weight of mouse PlGF was found to be about 30 kDa. DiPalma *et al.* (1996) pointed out that the mouse PlGF sequences showed 91% identity to the rat PlGF sequences. Although the murine PlGF protein was found with a relative molecular mass about 30 kDa, its molecular mass would still depend on the amount of N-glycosylation on its 17.8 kDa native protein (DiPalma *et al.*, 1996). So, different amount of glycosylation would be one possible explanation for this size discrepancy between the reported size and the one found in the present study. Furthermore, differences in the protein sequences between rat and mouse would be another reason for the size discrepancy.

The molecular weights of VEGF-B_{186} and VEGF-B_{167} isoforms were found to be 35 kDa and 24 kDa respectively in the present study. According to the studies by Olofsson *et al.*, the mouse and human VEGF-B genes were almost identical (Olofsson *et al.*, 1996a, b). Their molecular masses were found to be 32 kDa and 21 kDa respectively for VEGF-B_{186} and VEGF-B_{167} in mouse (Olofsson *et al.*, 1996a, b). Both the secreted forms of VEGF-B (VEGF-B_{186} and VEGF-B_{167}) were found to be modified by O-linked or N-linked glycosylation (Olofsson et al., 1996a, b). So, the size discrepancy between the reported sizes and those found in the present study could be resulted from differences in the protein sequences between rat and mouse, and amount of O-linked or N-linked glycosylation.

For VEGF-C, molecular weight of its peptide was found to be 27 kDa in the Western blotting. According to Kukk *et al.* (1996), VEGF-C was reveled as a secreted polypeptide of 30 to 32 kDa in weight. Kukk *et al.* (1996) also demonstrated that the pattern of proteolytic processing of VEGF-C in mouse and human was nearly identical. So, discrepancy between the reported size and the size found in the present study could be resulted from different protein sequences between rat and mouse, level of proteolytic processing and amount of glycosylation.

Similar to VEGF-C, VEGF-D is synthesized as a preproprotein requiring proteolytic processing in both the N- and C-terminal regions for activity, and the fully

processed growth factor is a non-covalent dimer (Orlandini *et al.*, 1996). The 37 kDa peptide reveled in the Western blotting was actually the unprocessed precursor of VEGF-D. Moreover, according to the study by Orlandini *et al.* (1996), the 23 kDa and 16 kDa peptides would probably be two proteolytic processed forms of VEGF-D, though the discrepancy could be because of the different protein sequences between mouse and rat.

Presence of VEGF-C and VEGF-D in the Leydig cells indicates their possible involvement in the development of the testicular lymphatic vessels through binding to VEGFR-3 and regulating vascular functions through VEGFR-2 present on testicular vascular endothelium.

Similar to VEGF-A, VEGF-D was also immunolocalized to vascular smooth muscle cells. VEGF-D has been shown to be angiogenic to endothelial cells, although it is less potent than VEGF-A (Achen *et al.*, 1998; Marconcini *et al.*, 1999; Orlandini *et al.*, 1996). This may imply that VEGF-D is also involved in regulating the growth and maintenance of the testicular blood vessels.

In this study, we demonstrated the existence of VEGF-B and PlGF that they may form heterodimers with VEGF-A when co-expressed in the same tissue or cell (Cao *et al.*, 1996; DiSalvo *et al.*, 1995; Olofsson *et al.*, 1996a, b). Such overlapping expression of VEGF-A, VEGF-B and PlGF in the testis suggests that

VEGF-A/VEGF-B or VEGF-A/PlGF heterodimers may formed naturally within the testis to affect the bioavailability of VEGF-A and its action on vascular endothelial cells and other non-endothelial cell types.

The present study further demonstrated that destroying the Leydig cells by ethane dimethane sulphonate (EDS) treatment decreased the levels of VEGF-A_{164} and VEGF-A_{120} isoforms, VEGF-B_{186} and VEGF-B_{167} isoforms, VEGF-C peptides, but increased the levels of VEGF-D (mRNA and 16 kDa isoform) and PlGF. These results suggest that Leydig cells are representing the primary source of VEGF-A_{164} and VEGF-A_{120} isoforms, VEGF-B_{186} and VEGF-B_{167} isoforms and VEGF-C. On the contrary, VEGF-A_{188} isoform, VEGF-D 37 kDa, 23 kDa and 16 kDa isoforms and PlGF are not mainly produced by Leydig cells.

VEGF-D was recently shown to be androgen-repressible (Ezer & Robaire, 2003). So, removal of Leydig cells in the treatment caused decline in circulating androgen that in turn induced an increase in VEGF-D 16 kDa isoform from other testicular cells and tissue (probably the vascular smooth muscle cells and Sertoli cells as illustrated in the immunohistochemical staining study). In other words, there were other major sources of VEGF-D 16 kDa and PlGF peptides besides the Leydig cells, and / or their expression was repressed by androgen.

The vascular effects of hCG on the testis has been well-documented. In adult

rats, the testes respond to a single injection of hCG with a decrease followed by an increase in blood flow, and an inflammation-like reaction of polymorphonuclear leukoctes infiltration and vascular permeability increase (Setchell & Sharpe, 1981; Widmark *et al.*, 1986). The last effect results in the accumulation of interstitial fluid which could explain why the testis weight of these animals was significantly increased at 2 days post-hCG. Since no LH/hCG receptors have been found in testicular blood vessels or on the surface of vascular endothelial cells. Thus the vascular effects of hCG are likely to have been produced indirectly through the release of other mediators from within the testis.

Chronic suppression of Leydig cells by subcutaneous testosterone-filled implants induced a declining expression on VEGF-A mRNA, VEGF-A_{164} protein isoform, VEGF-B_{167} protein isoform, VEGF-C mRNA and protein. In contrast to EDS treatment, Leydig cell suppression did not result in significant decreases in the levels of VEGF mRNA levels. Only VEGF-A_{164} peptide levels fell in both EDS treatment and Leydig cell suppression. Besides, results on VEGF-B_{167} isoform and VEGF-C correlated with the findings in EDS treatment quite well and suggested that Leydig cells could be the source of these peptides.

The present study confirms that a single injection of 100 IU hCG induces an increased synthesis of VEGF-A_{164} in testes of normal rats and those receiving

testosterone implants in 2-days post-hCG, which is in line with the observation of Haggstrom Rudolfsson *et al.* (2003). Leydig cells would be the major cell type responsible for producing such physiological response because of, first, hCG (a LH agonist) binds selectively to Leydig cells; second, Leydig cell was proved to be the major source of VEGF-A_{164} in the previous EDS treatment study. Besides, we have further demonstrated that injection of hCG induced increases in VEGF-B_{186} mRNA and protein, VEGF-C mRNA and VEGF-D mRNA and protein. So, expression of the above VEGF family members was shown to be hormonally regulated by hCG.

To summarize all the findings in the present study, two points can be drawn. First, there were discrepancies in the changes of VEGF-related peptide mRNA and protein levels following EDS treatment (removal of Leydig cells and androgen) and suppression of Leydig cell function using T-implants. Second, angiogenic factor(s) responsible for mediating hCG-induced increase in endothelial cell proliferation remained unclear. Consistent increases were observed only for VEGF-A_{164}, but the absolute levels did not appear to correlate with the degree of endothelial cell proliferation. Based on these findings, further studies will be required to examine other parameters, first, the metalloproteases or heparinase which may play a role in the release of these VEGF-related peptides from their association with heparan sulphate proteoglycan on cell surface and extracellular matrix; second, the expression

of other pro-angiogenic or anti-angiogenic factors.

In conclusion, expression of various new VEGF family members, including PlGF, VEGF-B, VEGF-C and VEGF-D, was demonstrated in the testis (mainly found in Leydig cells and Sertoli cells). Leydig cell was demonstrated to be a major source of VEGF-A_{164} and VEGF-A_{120} isoforms, VEGF-B_{186} and VEGF-B_{167} isoforms. Expression of VEGF-D (mRNA and 16 kDa isoform) and PlGF was demonstrated to be androgen repressible. Besides, expression of VEGF-A_{164} was demonstrated to be hormonally regulated by hCG.

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